Effect of temperature, water-phase salt and phenolic contents on Listeria monocytogenes growth rates on cold-smoked salmon and evaluation of secondary models.

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Abstract

Salting and smoking are ancient processes for fish preservation. The effects of salt and phenolic smoke compounds on the growth rate of *L. monocytogenes* in cold-smoked salmon were investigated, through physico-chemical analyses, challenge tests on surface of cold-smoked salmon at 4°C and 8°C, and a survey of the literature. Estimated growth rates were compared to predictions of existing secondary models, taking into account the effects of temperature, water phase salt content, phenolic content, and additional factors (e.g. pH, lactate, dissolved CO_2). The secondary model proposed by Devlieghere *et al.* [Food Microbiol. 18 (2001) 53] and modified by Giménez and Dalgaard [J. Appl. Microbiol. 96 (2004) 96] appears appropriate. However, further research is needed to understand all effects affecting growth of *L. monocytogenes* in cold-smoked salmon and to obtain fully validated predictive models for use in quantitative risk assessment.

Keywords: Fishery products. *L. monocytogenes*. Predictive microbiology. Microbial exposure assessment. Validation criteria. Phenolic compounds.

1. Introduction

Numerous recent or in-process risk assessments have concerned *Listeria monocytogenes* in sliced and vacuum packed cold-smoked salmon (Buchanan, 1997; Lindqvist and Westöö, 2000; FSANZ, 2002; Beaufort *et al.*, 2002; FDA, 2003; FAO/WHO, 2004). This abundance is clearly justified by the sanitary and economic importance of this issue, but it may also be explained by the relatively good availability of data. *L. monocytogenes* is indeed a well known foodborne pathogen which has been extensively studied since the first major recognised outbreak in the early 1980s (Schlech *et al.*, 1983). Presence and potential growth of this pathogen in cold-smoked salmon has been widely reviewed (Ben Embarek, 1994; Rorvik, 2000; Ross *et al.*, 2000). However, as noted in most risk assessment reports, there are still research needs to better characterize the contamination data and to improve and validate the tools of predictive microbiology to predict growth of *L. monocytogenes* in cold-smoked salmon.

Predictive microbiology aims to predict microbial behaviour in food over time as a function of different influencing parameters. For a review of such models, see McKellar and Lu (2004). Briefly, primary models describe the evolution of a population of micro-organisms over time under certain conditions whereas secondary models describe how the primary model parameters, e.g. the lag time (*lag*) and the growth rate (μ_{max}), vary with environmental conditions. They are typically based on data generated in liquid laboratory culture media, whereas they aim to predict growth in food products. Validation is then an important issue. Model validation can be defined as demonstrating the accuracy of the model for a specified use. Ross (1996) and Baranyi *et al.* (1999) proposed criteria to measure the performance of a model, i.e. their reliability when compared to independent "real-world" data, obtained in inoculated food products (challenge tests) or even in naturally-contaminated products (storage trials), and not used to generate the

model.

Dalgaard and Jorgensen (1998) provided an extensive comparison of existing secondary models for *L. monocytogenes*, on the basis of 100 literature challenge tests in different seafood products (including 26 in cold-smoked salmon) and 13 storage trials in cold-smoked salmon. As stated by the authors, one of the limitations was that the inhibiting effect of smoke components was not taken into account, both because no adequate secondary model was available at that time and because the concentration of smoke components was not measured in products used for challenge tests. The antimicrobial activity of smoke is generally attributed to the phenolic fraction, even if no relationship between concentration of phenolic compounds and growth inhibition has clearly been established (Thurette *et al.*, 1998; Niedziela *et al.*, 1998; Suñen *et al.*, 2001, 2003; Lebois *et al.*, 2004). Since this study, Augustin and Carlier (2000a, b) have proposed two secondary models taking into account the phenolic content, and Giménez and Dalgaard (2004) have modified two other secondary models to include this phenolic effect.

The objective of the present study was (*i*) to further investigate the physicochemical characteristics of cold-smoked salmon, including the phenolic content, on the basis of a specific survey, and of former similar studies (Leroi *et al.*, 2001; Espe *et al*., 2004), and (*ii*) to characterize how they affect growth of *Listeria monocytogenes*, on the basis of specific challenge tests and of literature data (Peterson, *et al.*, 1993; Pelroy, *et al.*, 1994; Rosso *et al.*, 1996; Niedziela, *et al.* 1998; Giménez and Dalgaard, 2004; Lakshmanan and Dalgaard, 2004). The four recently proposed secondary models taking into account the concentration of phenolic compounds (Augustin and Carlier, 2000a, b; Giménez and Dalgaard, 2004), and 7 additional secondary models, were compared and evaluated.

2. Materials and methods

2.1. Physicochemical analyses of French commercial products

Eight French companies each provided five randomly sampled commercial products, which were received frozen and vacuum-packaged. Water, salt and phenolic contents and pH were measured, according to procedures described by Leroi *et al.* (2000, 2001). Salt contents (in g/100g) were divided by water contents to obtain water phase salt (WPS) in g/100 ml.

Similar results published by Leroi *et al.* (2001) and Espe *et al*. (2004) are also presented. Statistical *t*-tests were performed to compare the new results with the previous ones of Leroi *et al.* (2001), with $\alpha = 0.05$.

*2.2. Challenge tests (*L. monocytogenes*) in 5 specific products*

Five French companies were asked to produce a specific batch of cold-smoked salmon, achieving realistic physicochemical goals (high, medium or low levels of smoking and salting). The five batches were denoted A to E. Three batches (A, B, C) had been manually dry-salted, one batch (E) had been mechanically dry-salted, whereas batch D underwent both a mechanic dry salting and injection. Only salt was added to the raw fish (i.e. no nitrites, no sugar). They all had been cold-smoked (A: 22-24°C, B: 23°C, C: not communicated, D: 25-27°C, E: 24-26°C) in kilns, using either beech wood (batches A, B, D, E), or a mixture of woods, including mostly beech and oak (batch C). Batches A and C were produced in artisanal plants, whereas batches B, D, and E were produced in industrial plants. Samples were received from the plants frozen and vacuum-packaged.

For each batch, two 20-slice sub-batches were thawed overnight at 2°C and an 89 mm-diameter disk was excised from each fish slice. Weights of the disks ranged from 15 g to 20 g. The disks of one sub-batch were further inoculated with *L. monocytogenes* and used for a challenge test at

4°C, while non-inoculated off-cuts of the same sub-batch were used for a storage trial at 4°C, whereas the disks and off-cuts of the other sub-batch were used for a challenge test and a storage trial at 8°C. Last, all remaining off-cuts of one batch were pooled and the pool was analysed for pH, salt content, and phenolic compounds according to the procedures detailed above. This pooling was chosen to reach some confidence in estimating the average physicochemical characteristics of each batch, but did not enable us to observe any within-batch physicochemical variability (neither between the sub-batches, nor within a sub-batch).

Strain TQA 061, isolated in the laboratory from commercial cold-smoked salmon, and stored at -24°C in a glycerol-containing medium, was used for inoculation of the disks. Prior to challenge testing, the content of one cryotube was thawed, and it was 1/100 diluted in tryptone soya broth (AES, Combourg, France) and cultured 4 days at 10°C. This first preculture in early stationary phase was 1/100 diluted in tryptone soya broth and cultured 3 days at 10°C. This second preculture in early stationary phase was 1/1000 diluted in tryptone salt (AES), to obtain the inoculum suspension, at a level of 2.10^6 cfu per millilitre. A 0.1-ml volume of this inoculation suspension was spread onto each disk, which was then folded, so that the inoculum was sandwiched between the two layers. The folded disks were vacuum-packaged, using a chamber machine Multivac A300/16 (Multivac, Lagny-sur-Marne, France), in polyamide/polyethylene (PA/PE) 30 µm/70µm film (Euralpack, Saint Pierre du Perray, France), with low transmission rates: 30 to 40 $cm^3.m^{-2}.day^{-1}.bar^{-1}$ for O_2 (23°C, 75% relative humidity), 90 $cm^3.m^{-2}.day^{-1}.bar^{-1}$ for CO_2 (23°C, 75% relative humidity), 2.5 $g.m^{-2}.day^{-1}$ for H_2O (23°C, 85% relative humidity). For each batch, twenty inoculated 89-mm disks were stored at 4°C±1°C, whereas the twenty other disks were stored at 8°C±1°C, with continuous temperature monitoring. After various intervals up to 55 days, packages were 1/10 diluted in tryptone salt, and homogenized with a

125 stomacher. *L. monocytogenes* was enumerated by plating onto Palcam agar (AES) appropriate
126 dilutions of the disks in tryptone salt. Plates were incubated 48h±2h at 37°C±1°C. Cell counts
127 were calculated per square centimetre of salmon surface, so that population densities of
128 *L. monocytogenes* were expressed as log cfu/cm^2. The theoretical initial contamination,
129 calculated from the contamination level for the inoculation suspension, is 3.5 log cfu/cm^2, which
130 is approximately equivalent to 4 log cfu/g.

131 *2.3. Storage trials (naturally occurring food flora) in the same 5 products*

132 To study the growth of the mesophilic food flora, storage trials of each batch were performed.
133 From the non-inoculated crushed off-cuts of each batch, 36 10g-packs were weighted, and
134 vacuum-packaged (as described above). Then 18 10g-packs were stored up to 55 days at 4°C,
135 and 18 10g-packs were stored up to 25 days at 8°C. After various intervals, packs were 1/10
136 diluted in tryptone salt, and homogenized with a stomacher. The mesophilic food flora was
137 enumerated by plating onto Plate Count Agar (AES) appropriate dilutions of the packs in
138 tryptone salt. Plates were incubated 3 days at 30°C. Cell counts were expressed as log cfu/g.

139 *2.4. Estimation of growth rates*

140 Each *L. monocytogenes* growth curve was fitted by the model of Baranyi and Roberts (1994) and
141 by the embedded model without lag-phase, using least-squares non linear regression. An *F*-test
142 was performed to compare both models, with $\alpha = 0.05$. In all cases but one (Batch E, 8°C), the
143 lag time was not significant and the model without lag phase was then selected. This is easily
144 explained by the history of the strain, as the preculture temperature was 10°C. The parameters of
145 the chosen model were also estimated using robust non linear regression, as detailed in Miconnet
146 *et al.* (in press). All calculations were performed with the software Matlab 6.5 (MathWorks).
147 When it was appropriate, the same estimation was performed for the food flora, obtained through

148 storage trials.

149 To validate the secondary models, additional growth rates of *L. monocytogenes* were obtained
150 from published challenge tests. Only products in which the phenolic content was either null or
151 measured were selected. Growth rates in cold-smoked salmon estimated by Giménez and
152 Dalgaard (2004) and Lakshmanan and Dalgaard (2004) were used as published by the authors.
153 Published graphs in smoked salmon (Rosso *et al.*, 1996), and in cold-process (not smoked)
154 salmon (Peterson, *et al.*, 1993; Pelroy, *et al.*, 1994; Niedziela, *et al.* 1998) were scanned and
155 individual points digitalised. Growth rates were estimated using the same procedure as
156 previously described.

157 *2.5. Prediction of* L. monocytogenes *growth rates using secondary models*

158 Four secondary models, taking into account at least the effects of temperature, water activity
159 (calculated from the NaCl content) and phenolic content, were used for predictions:

160 • Model 1, a cardinal model developed by Augustin and Carlier (2000a) on the basis of
161 1437 literature growth rates, both in broth and in challenge tests;
162 • Model 2, a cardinal model including interactions between factors (Augustin and Carlier,
163 2000b), based on the same data set;
164 • Model 3, a square-root model, developed by Tom Ross, used by FAO/WHO (2004), and
165 modified by Giménez and Dalgaard (2004) to take into account the phenolic effect;
166 • Model 4, a square-root model, developed by Devlieghere *et al.* (2001) and similarly
167 modified by Giménez and Dalgaard (2004).

168 Additional models, not taking into account the phenolic content, were also considered to
169 calculated the validation criteria:

170 • Models 1', 2', 3' and 4', similar to models 1, 2, 3, and 4, without the phenolic effect,

- Model 5, a polynomial model, based on growth curves in broth, used in Pathogen Modelling Program, a software developed by USDA (2001),
- Model 6, a polynomial model, based on growth curves in broth, used in Growth Predictor, a software developed by IFR (2004),
- Model 7, a square-root model, developed by FDA (2003) on the basis of 29 literature growth curves in smoked fishery products, not taking into account the physicochemical factors.

Models were applied as originally defined by their authors. The water activity, a_w, was calculated from WPS by the equation used by Augustin and Carlier (personal communication) and Giménez and Dalgaard (2004):

$$a_w = 1 - 0.0052471\ WPS - 0.00012206\ WPS^2 \quad (1)$$

When needed in the model and not measured, the water-phase lactate (WPL) level of cold-smoked salmon was assumed to be 90 mM (Tienungoon *et al.*, 2000), which is equivalent to a concentration of sodium lactate (NaL) at 1%. All other concentrations of inhibiting compounds (including dissolved CO_2) were assumed to be null. As Model 7 provides a distribution of predicted growth rates at each temperature, the average of the distribution was chosen for validation.

2.6. Validation criteria

Two criteria, proposed by Ross (1996), were used to compare these models:

- the accuracy factor, which expresses the accuracy of the model predictions (1 if all predictions are equal to the observations),
- the bias factor, which expresses the overall bias (> 1 for a fail-safe model, < 1 for a fail-dangerous model, 1 for an unbiased model).

194 The null growth rates predicted by models 1 and 2 were by convention replaced by 0.01day^{-1}, to
195 obtain numerical values of the validation criteria.

196 **3. Results and discussion**

197 *3.1. Physicochemical characteristics*

198 Forty French commercial products were surveyed and analysed for pH, salt contents, water
199 contents, water-phase salt contents, and phenolic contents (see table 1). Results were compared
200 with those of Leroi *et al.* (2001), on 13 French commercial products, supposed to be
201 representative of the French traditional production and Espe *et al*. (2004), on 48 French
202 commercial products, produced by four commercial smoking-houses. Water and salt contents
203 were very similar with those observed by Leroi *et al.* (2001). Significant differences were
204 observed between both studies regarding two factors: pH and phenolic contents. Indeed, Leroi *et*
205 *al.* (2001) measured "initial" pH in early shelf life, whereas pH-values were measured later in the
206 shelf life in the present study, which may explain the slightly lower results. Last, Leroi *et al.*
207 (2001) observed lower phenolic contents, as low as 0.27 mg/100g, and none above 1.1 mg/100g.
208 This difference may be explained by an unexpected sampling or experimental bias or could
209 reflect a recent evolution of the French market. Statistical comparisons could not be performed
210 using results of Espe *et al*. (2004), as raw results had not been published, but results appeared
211 close to the ones of the present survey.
212 The measured physicochemical characteristics of the 5 specific products used for challenge tests
213 (see Table 1) were all within the ranges of those of the commercial products (at least from one of
214 the three surveys, see Table 1). However, one batch (C) appeared relatively lightly salted. The
215 phenolic contents of two batches (B and E) were relatively low, whereas batch A had a relatively

high phenolic content (2 mg/100 g). Last, the initial pH values were similar to those of Leroi *et al.* (2001).

3.2. Growth of L. monocytogenes *in specific products*

For each batch A to E, two challenge tests were performed: one at 4°C, and the other one at 8°C. Figure 1 presents the ten observed growth curves of *L. monocytogenes*. It has to be underlined that such growth curves were obtained under particular laboratory conditions (specific products, high inoculum levels, preculture in culture broth...) and do not aim to simulate realistic natural contamination. Indeed, storage trials, monitoring of naturally contaminated products, are the only experiments that really enable us to fully describe this state of natural contamination. Concerning specifically the growth rate (μ_{max}), it is usually accepted that challenge tests, whatever the inoculum level, are an adequate and useful approximation of storage trials, whereas it is far more discussed for the two other parameters of primary growth models, the lag time (*lag*) and the maximum population density (*MPD* or N_{max}). For a further discussion of this, see Gnanou-Besse *et al*. (submitted).

These extreme physico-chemical conditions of batches A to E were intentionally selected to better characterize the effects of salting and smoking on the growth rate of *L. monocytogenes* at two temperatures. In one combination (batch A, 4°C), less than three generations of *L. monocytogenes* were observed within 55 days. At 8°C, this highly smoked batch was associated to the slowest growth. These results confirm that the impact of phenolic compounds at a very high level, 2 mg/100g, superior to the levels usually encountered on the market.

These growth curves were modelled by a primary model, using the classical least-squares criterion or an alternative robust criterion. Results of the two methods were close (Table 2) and those of the classical least-squares regression were used for further discussion.

239 The similarity of growth rates at 4°C and 8°C of batch B was unexpected. This unexpected result
240 might be (at least partly) due to estimation uncertainty. Note that the obtained growth curves,
241 both at 4°C and 8°C, are far from ideal exponential growth curves. The uncertainty on the
242 estimation of growth rates is then high. As discussed in Miconnet *et al*. (in press), surface growth
243 curves are often less satisfactory than crushed growth curves, due to an heterogeneity between
244 packs, which are not homogenised in the 1st case, whereas they are in the latter case.
245 This unexpected result of batch B could also be explained by the within-batch variability.
246 Indeed, there could have been a difference between the sub-batch used for the growth curve at
247 4°C and the sub-batch used for the growth curve at 8°C.
248 Moreover, at 4°C, the growth rate in batch B is higher than in batch E, and the opposite is
249 observed at 8°C, whereas batches B and E have similar physicochemical characteristics. This
250 may be explained by the estimation uncertainty discussed above. It also illustrates the fact that
251 the measured physicochemical characteristics probably do not account for all the between-
252 batches and within-batch variability.

253 *3.3. Growth of the food flora in specific products*

254 Figure 2 presents the growth curves of the mesophilic food flora in storage trials at 4°C and 8°C.
255 The two artisanal batches, denoted A and C, had relatively high initial plate count numbers. It
256 was then possible to estimate, at least roughly, the growth rates of the mesophilic food flora.
257 This estimation is very approximate as storage trials are less appropriate than challenge tests for
258 such fittings. Moreover the growth rate is usually defined for a single species, whereas it is used
259 in this case for a mixture of species. The least-squares estimations were: 0.27 day^{-1} (batch A,
260 4°C), 0.58 day^{-1} (batch A, 8°C), 0.96 day^{-1} (batch C, 4°C), and 0.86 day^{-1} (batch C, 8°C). The
261 robust estimations were close: 0.27 day^{-1} (batch A, 4°C), 0.63 day^{-1} (batch A, 8°C), 0.91 day^{-1}

(batch C, 4°C), and 0.89 day^{-1} (batch C, 8°C). The fact that the growth is at both temperatures faster in the lightly salted batch C than in the heavily smoked batch A can easily be explained by the physicochemical difference between the two sub-batches. Indeed, most indigenous microbial flora are probably, at least partly, inhibited by salt and phenolic compounds. On the contrary, it was less expected that the microbial growth in batch C at 4°C could be as fast (or even faster) than at 8°C in the same batch. This might be partly explained by the hypothesis that some species present in the cold-smoked salmon could have an optimal temperature close to these temperatures (but would also be able to form colonies on PCA at 30°C in 3 days).

For these two batches, and especially for batch A, the population levels reached by the food flora in storage trials is close or even higher than the contamination levels of *L. monocytogenes* in challenge tests. Then, it is possible that, in these two batches, the observed growth of *L. monocytogenes* had been influenced by the simultaneous growth of a non-neglectable or even predominant background flora. It has often been observed that the major interaction observed in cold-smoked salmon between the background flora (among which the lactic acid flora tends to be predominant) and *L. monocytogenes* is a competition, the so-called Jameson effect, with a simultaneous deceleration of all populations (see Buchanan *et al*., 1997, 1999; Dalgaard and Jorgensen, 1998; Ross *et al*., 2000; Cornu, 2001; FAO/WHO, 2004; Giménez and Dalgaard, 2004; Nilsson *et al*., 2005). Even if the data are not appropriate to detect such an effect, the deceleration of *L. monocytogenes* at a relatively low level (ca. 10^6 cfu/cm^2), in batch A at 8°C, after ca. 20 days, could be explained by the simultaneous deceleration of the food flora (observed in storage trials after ca. 17 days at 8°C).

This Jameson effect only impacts the maximum population density of *L. monocytogenes* and not its growth rate, which was the major focus of this paper. Then, we assume that the estimations of

285 the growth rates in these batches were not influenced by the background flora. On the contrary,
286 the observed maximal population densities observed by *L. monocytogenes* may be lower than
287 those obtained in absence of this predominant background flora.
288 For the three industrial batches, denoted B, D, and E, the initial population was so low, that most
289 packs could not be enumerated using standard techniques (with a quantification threshold at 10
290 cfu/g). It is then impossible for these batches to estimate the growth rates. However, in the case
291 of batch B, it cannot be excluded that the growth at 4°C could be close to the growth at 8°C (as
292 enumeration results are similar at the 18th day). This could confirm that there was a difference
293 between the physicochemical characteristics of the sub-batch used at 4°C and the sub-batch used
294 at 8°C. This could also be explained by species with a low optimal temperature, as for the food
295 flora of batch C discussed above. For these three batches, *L. monocytogenes* was strongly
296 predominant in the challenge tests and we can then exclude that any competition effect occurred
297 during the experiments. However, as realistic initial contamination levels of *L. monocytogenes*
298 are very low (see Beaufort *et al.*, submitted), competition should be taken into account when
299 predicting the evolution of *L. monocytogenes* in naturally contaminated products, even for these
300 batches with a relatively initial level in background flora.

301 *3.4. Comparison and validation of* L. monocytogenes *secondary models*

302 Published secondary models were evaluated in this study. Predicted growth rates are compared
303 with estimated growth rates of the present study in Table 2. Predictions of models 1 and 2 were
304 much lower than the observations (*i.e.* fail-dangerous). Predictions of models 3 and 4 were more
305 consistent with estimated growth rates, even if model 3 tended to be fail-dangerous at 4°C. The
306 estimated growth rate for batch E at 8°C, 1.63 day^{-1}, appears relatively high when compared with
307 the other estimations and with the predictions. For this specific growth curve, the lag time was

significantly non null, but its biological significance can be questioned. Indeed, with a null lag time, the estimated growth rate is 0.92 day^{-1}, which appears more consistent with the predictions. This example is an indication that the estimation procedure of the growth parameters is much more complex in the case of challenge tests, than in the case of curves in broth.

For the sake of comparison, models 1, 3 and 4 were rewritten into a unified five-parameter equation:

$$\mu = \mu_{ref} \cdot \frac{(T - T_{min})^2}{(T_{ref} - T_{min})^2} \cdot \frac{(WPS - WPS_{max}) + 0.02326\left(WPS^2 - WPS_{max}^{\ 2}\right)}{(WPS_{ref} - WPS_{max}) + 0.02326\left(WPS_{ref}^{\ 2} - WPS_{max}^{\ 2}\right)} \cdot \frac{(P_{max} - P)^k}{(P_{max} - P_{ref})^k} \quad (2)$$

where T_{min} is the minimal temperature, WPS_{max} the MIC-value for WPS, calculated from the minimal a_w of each model, using Equation (1), P_{max} the phenolic MIC-value, k equals 1 or 2, and μ_{ref} the predicted growth rate for a reference cold-smoked salmon at a reference temperature, *i.e.* the prediction of the model for the following conditions: $T = T_{ref} = 5°C$; pH = 6.20; WPS_{ref} = 5.0%; $P = P_{ref}$ = 1.0 mg/100g = 10 ppm; WPL = 8000 ppm = 90 mM, corresponding to NaL = 1%; CO_{2diss} = 0 ppm.

The reference values for pH, WPS and P, were arbitrarily set at rounded average values (see Table 1), whereas the choice of the reference value for lactate concentrations was based on Tienungoon *et al.* (2000).

Table 3 presents the parameters of Models 1, 3 and 4. Model 2 could not be rewritten in such a unified form, moreover it predicts a null growth rate for the reference cold-smoked salmon at the reference temperature. This presentation was conceived to compare models. Thus, the very low phenolic MIC-value (P_{max}) of model 1 is sufficient to explain why model 1 was highly fail-dangerous in smoked products, whereas it was much more appropriate and even slightly fail-safe in non-smoked products, in which the phenolic effect was not modelled. The minimal

temperature of model 3, +0.88°C, appears relatively high, which may explain why this model behaves better at 8°C than at 4°C.

Equation (2) was also designed to enhance simpler use of these models. When no information concerning the salt content is available, the term describing its effect can simply be omitted. The water phase salt content is then assumed to be 5%. Similarly, if the term describing the effect of phenol is omitted, the phenolic content is assumed to be 1 mg/100g.

Table 4 presents the validation criteria based on different sets of growth rates: the 10 challenge tests on cold-smoked salmon of the present study, the 9 challenge tests on/in cold-smoked salmon taken from literature, and the 22 challenge tests in cold-process non-smoked salmon. Eleven secondary models were tested. Among the four models taking into account the phenolic effect, model 4 was the most accurate model (lowest *Af*-value) on each data set. It was slightly biased, in a fail-safe way, (*Bf*>1) but such a bias is usually preferred to a fail-dangerous bias. Model 4 is then a good candidate to take into account the effect of all physicochemical factors, including the phenolic content, on growth rates of *L. monocytogenes*.

However, satisfactory validation criteria were also obtained with some other models, especially with model 7. As this model was directly built from growth rates estimated in challenge tests, *Bf*-values close to 1.0 were expected and were indeed obtained. More surprisingly, the *Af*-values obtained with this approach, in which only the temperature effect was modelled, were close or better than the *Af*-values obtained with models taking into account the physicochemical factors, such as model 4.

Thus, the described between-product physicochemical variability does not appear sufficient to fully explain the between-curve variability of growth rates. Our description of the between-product physicochemical variability may be improved. Thus, the effect of organic acids was only

353 taken into account through the initial pH, whereas the production of lactic acid by the
354 background flora could have been specifically considered. The measurement of WPS is probably
355 not sufficient to study the water activity, as sucrose, measured by Espe *et al*. (2004) in French
356 products, or other solutes may also lower it. The within-batch variability, e.g. the variability of
357 the WPS due to this more or less equal repartition of the salt (which could depend on the salting
358 method), was not considered in this study but could have a great impact, as suggested by some
359 unexpected results. Last, Brocklehurst (2003) reviews numerous studies which demonstrate that
360 the microstructure of the food impacts the microbial growth.
361 In a broader context, additional preservatives, which are forbidden in France and were not used
362 in the batches selected for these experiments, may be taken into account. For example, when
363 they have been used, nitrites had a significant effect (Pelroy *et al*., 1994).
364 Then, additional sources of between-product and within-product variability have still to be
365 investigated, before a full validation of secondary models based on physicochemical
366 characteristics. Investigation of secondary models based on a alternative description of the
367 variability could also be valuable.

368 *3.5. Phenolic effect*

369 The highly fail-dangerous characteristic of models 1 and 2 can be easily explained, as Augustin
370 and Carlier (2000a, b) based their estimations of the phenolic MIC-value on experimental results
371 in which a phenolic concentration of 1.25 mg/100 ml in broth was inhibitory for
372 *L. monocytogenes* (Membré *et al.*, 1997), whereas a concentration of 2 mg/100 g is not, at least
373 at 8°C, in our results. This apparent contradiction may be explained by a difference between the
374 behaviour of phenolic compounds in broth *versus* fish. The solubility of these compounds in the
375 water phase of a fatty fish is rather unknown, but it can be expected to be low and dependent on

the nature of smoke and the temperature. Second, the phenolic concentration is probably not sufficient to assess the antimicrobial activity of smoke. Suñen *et al.* (2001) observed growth of *L. monocytogenes* at 5°C in broth at a phenolic concentration as high as 10.75 mg/100 ml, but no growth at a phenolic concentration of 2.3 mg/100 ml, with another smoke extract.

Last, the between-strain variability in sensitivity to phenolic compounds should also be considered. According to results of Thurette *et al.* (1998), a concentration of 1.1 mg/100 g in cold-smoked fish at 4°C was inhibitory for their reference strain but not for a cocktail of three strains, including one isolated from smoked fish.

Thus, results appear relatively controversial. The nature of the smoke, either wood smoke or liquid smoke, and the analytical procedure to measure phenolic concentrations differ from one study to another and this may add confusion. Even if the MIC-value chosen by Giménez and Dalgaard (2004) appears satisfactory on the basis of the results presented in this paper, numerous questions raised regarding the solubility of phenolic compounds, the between-strain variability, and the impact of non-phenolic smoke have still to be discussed.

4. Conclusion

Physiochemical characteristics of cold-smoked salmon, especially the contents in salt and phenolic compounds, affect growth rates of *L. monocytogenes*. Secondary models can be used to model these effects and, among the four tested models, the secondary model proposed by Devlieghere *et al.* (2001) and modified by Giménez and Dalgaard (2004) appeared the most appropriate one. However, it was obvious that the studied factors, including the phenolic content, were not sufficient to describe the whole variability of the behaviour of *L. monocytogenes* in cold-smoked salmon. Additional sources of uncertainty and variability affecting the growth rate should be considered, such as the between-strain variability and a between-product variability

399 which is not explained by the measured physicochemical factors.

400

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520 **List of Figures**

521

522 **Figure 1**. Growth curves of *L. monocytogenes* on cold-smoked salmon (log cfu/cm^2), after
523 surface-contamination of batches A (figure 1A) to E (figure 1E). Experimental results (grey
524 squares: 4°C, black squares: 8°C) were fitted by least-squares regression with the selected
525 primary model without lag (grey solid line: 4°C, black solid line: 8°C), and the Baranyi model
526 (black dotted line) for one curve (batch E, 8°C).

527 **Figure 2**. Growth curves of the mesophilic food flora in cold-smoked salmon (log cfu/g),
528 batches A (figure 2A) to E (figure 2E). Experimental results (grey squares: 4°C, black squares:
529 8°C) below a limit of quantification are represented at this limit with a vertical bar. For two
530 batches, A and C, growth curves were fitted by least-squares regression with the selected
531 primary model without lag (grey solid line: 4°C, black solid line: 8°C).

532

List of Tables

Table 1: Physicochemical characteristics (pH, salt content, water-phase salt content calculated from salt and water contents, and phenolic content) of cold-smoked salmon. For the present survey (40 French commercial products) and the survey of Leroi *et al.* (2001), mean, standard deviation, and extreme values are shown. Individual results are presented for the five specific batches (labelled A to E) used for challenge tests.

Table 2: Estimated and predicted growth rates (day^{-1}) of *L. monocytogenes* on cold-smoked salmon at 4°C and 8°C. Estimations were obtained fitting each growth curve using the chosen model (see Figure 1) and non-linear least-squares and robust regression (Miconnet *et al.*, in press). Predictions were obtained using four secondary models, and the physico-chemical characteristics of each product (see Table 1). Model 1: Augustin and Carlier (2000a). Model 2: Augustin and Carlier (2000b). Model 3: Ross (FAO/WHO, 2004), modified by Giménez and Dalgaard (2004). Model 4: Devlieghere et al (2001), modified by Giménez and Dalgaard (2004).

Table 3. Parameters of three models in a unified and simplified equation (see Equation (2) in the text). Model 1: Augustin and Carlier (2000a). Model 3: Ross (FAO/WHO, 2004), modified by Giménez and Dalgaard (2004). Model 4: Devlieghere et al (2001), modified by Giménez and Dalgaard (2004).

Table 4. Validation criteria of models 1 to 4 based on 41 growth rates of *L. monocytogenes*: 10 challenge tests on cold-smoked salmon in the present study (see Figure 1 and Table 2, the least-squares criterion and the model without any lag phase was selected for all curves), 9 challenge tests on/in cold-smoked salmon taken from literature (Rosso *et al.*, 1996; Giménez and Dalgaard, 2004; Lakshmanan and Dalgaard, 2004), and 22 challenge tests in cold-process

559 non-smoked salmon (Peterson, *et al.*, 1993; Pelroy, *et al.*, 1994; Niedziela, *et al.* 1998).
560 Model 1: Augustin and Carlier (2000a). Model 2: Augustin and Carlier (2000b). Model 3:
561 Ross (FAO/WHO, 2004), modified by Giménez and Dalgaard (2004). Model 4: Devlieghere
562 et al (2001), modified by Giménez and Dalgaard (2004). Model 1': Augustin and Carlier
563 (2000a), without phenolic effect ($P_{max} = \infty$). Model 2': Augustin and Carlier (2000b), without
564 phenolic effect ($P_{max} = \infty$). Model 3': Ross (FAO/WHO, 2004). Model 4': Devlieghere et al
565 (2001). Model 5: Pathogen Modeling program (USDA, 2001). Model 6: Growth Predictor
566 (IFR, 2004). Model 7: FDA (2003).
567
568

568 **Table 1.**
569
570

	pH	Salt content (g/100g)	WPS (g/100ml)	Water content	P = phenolic content (mg/100g)
40 commercial French products (present survey):					
Mean (SD)	6.02 (0.09)	2.85 (0.65)	4.62 (0.96)	61.3 (3.57)	0.99 (0.30)
[Min-Max]	[5.80-6.24]	[1.60-4.10]	[2.74-7.12]	[53.1-68.7]	[0.55-1.65]
13 commercial French products (Leroi et al., 2001):					
Mean (SD)	6.20 (0.07)	3.13 (0.56)	5.18 (0.90)	60.5 (3.08)	0.55 (0.26)
[Min-Max]	[6.09-6.30]	[2.21-4.29]	[3.76-7.19]	[57.3-68.0]	[0.27-1.08]
48 commercial French products (Espe et al., 2001):					
Mean (SD)	n.d.	2.62 (nd)	n.d.	62.5 (nd)	0.88 (nd)
[Min-Max]		[1.3-3.4]		[57.7-66.7]	[0.3-2.1]
5 specific batches, used for challenge tests:					
A	6.20	2.70	4.82	56.3	2.00
B	6.20	3.90	6.20	62.9	0.51
C	6.20	1.40	2.31	60.9	0.97
D	6.20	3.70	6.82	54.4	1.45
E	6.10	3.20	5.73	56.1	0.51

571
572

572 **Table 2.**

573

Conditions	Estimated growth rates (day^{-1})		Predicted growth rates (day^{-1})			
Batch, Temp.	Least-squares	Robust	Model 1	Model 2	Model 3	Model 4
A, 4°C	0.02	0.01	0	0	0.06	0.13
B, 4°C	0.50	0.48	0.12	0	0.14	0.29
C, 4°C	0.31	0.35	0.04	0	0.18	0.40
D, 4°C	0.29	0.31	0	0	0.07	0.15
E, 4°C	0.27	0.27	0.13	0	0.14	0.32
A, 8°C	0.27	0.30	0	0	0.31	0.30
B, 8°C	0.49	0.49	0.29	<0.01	0.72	0.67
C, 8°C	1.00	1.04	0.09	<0.01	0.93	0.94
D, 8°C	0.80	0.79	0	0	0.38	0.35
E, 8°C	1.63[a]	1.63	0.32	<0.01	0.74	0.74

574

575 [a]: the estimation of 1.63 day^{-1} for batch E at 8°C is obtained with the 4-parameter Baranyi

576 model. With a null lag time, the estimated growth rate is 0.92 day^{-1}.

577

577 Table 3.

Parameters	Model 1	Model 3	Model 4
μ_{ref} (day^{-1})	0.03	0.23	0.43
T_{min} (°C)	-2.7	0.9	-3.5
WPS_{min} (g/100mL)	13.1	11.6	10.7
P_{max} (mg/100g)	1.25	2.81	2.81
k	2	1	1

578

579

579 Table 4.

580

	Model 1[a]	Model 2[a]	Model 3	Model 4	Model 1'	Model 2'	Model 3'	Model 4'	Model 5	Model 6	Model 7
10 growth rates of the present study											
A_f	7.5	>10	1.9	1.7	4.2	> 10	2.0	2.0	3.1	2.3	1.8
B_f	0.1	<0.1	0.7	1.1	0.4	0.1	1.3	1.9	3.1	2.2	1.3
9 growth rates in cold-smoked salmon (literature)											
A_f	>10	>10	1.7	1.6	2.9	8.4	2.2	2.8	3.9	2.7	1.8
B_f	<0.1	0.1	1.1	1.6	2.9	0.1	2.1	2.8	3.9	2.7	1.8
10+9=19 growth rates in cold-salmon products (all sources)											
A_f	>10	>10	1.8	1.6	3.5	>10	2.1	2.4	3.4	2.5	1.8
B_f	0.1	<0.1	0.9	1.3	1.0	0.1	1.6	2.3	3.4	2.5	1.5
22 growth rates in salted (non-smoked) salmon (literature)											
A_f	1.7	5.2	1.6	1.5	1.7	5.2	1.6	1.5	2.3	1.5	1.5
B_f	1.6	0.2	1.0	1.5	1.6	0.2	1.0	1.5	2.3	1.5	0.8

581

582 [a] : For models 1 and 2, the criteria are not defined, as some predicted growth rates are equal to 0. To obtain

583 numerical values, null predictions were replaced by 0.01 day^{-1}

584

584

5 Figure 1

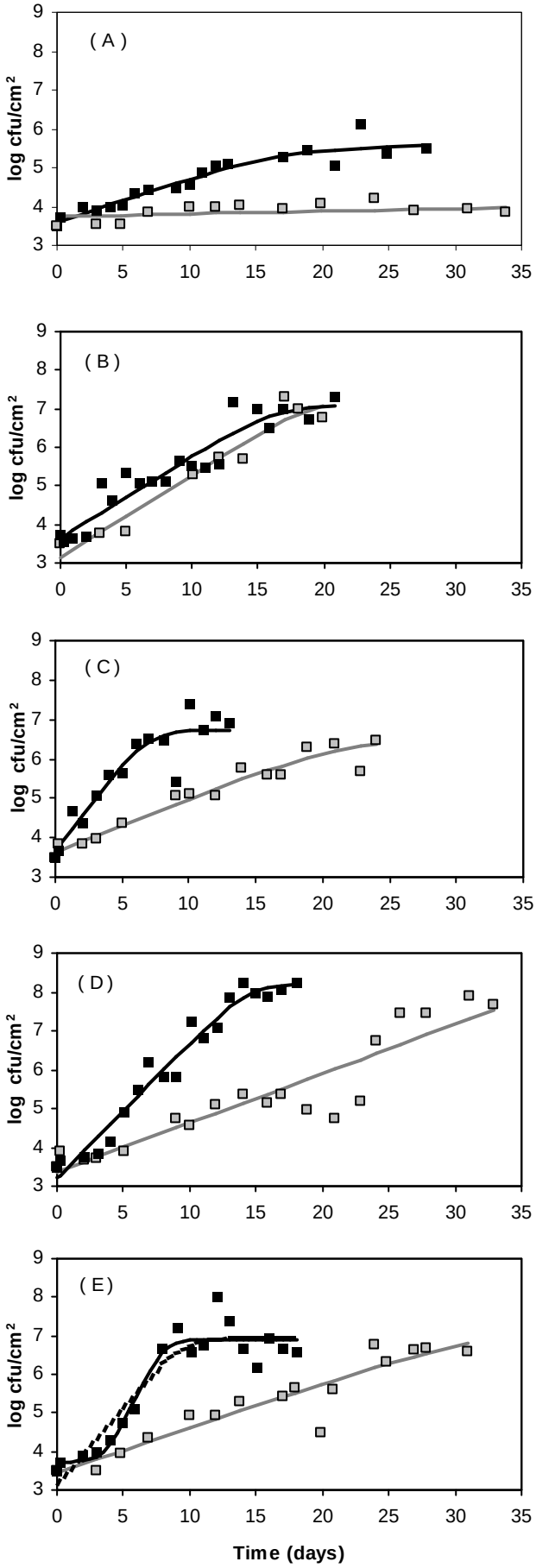
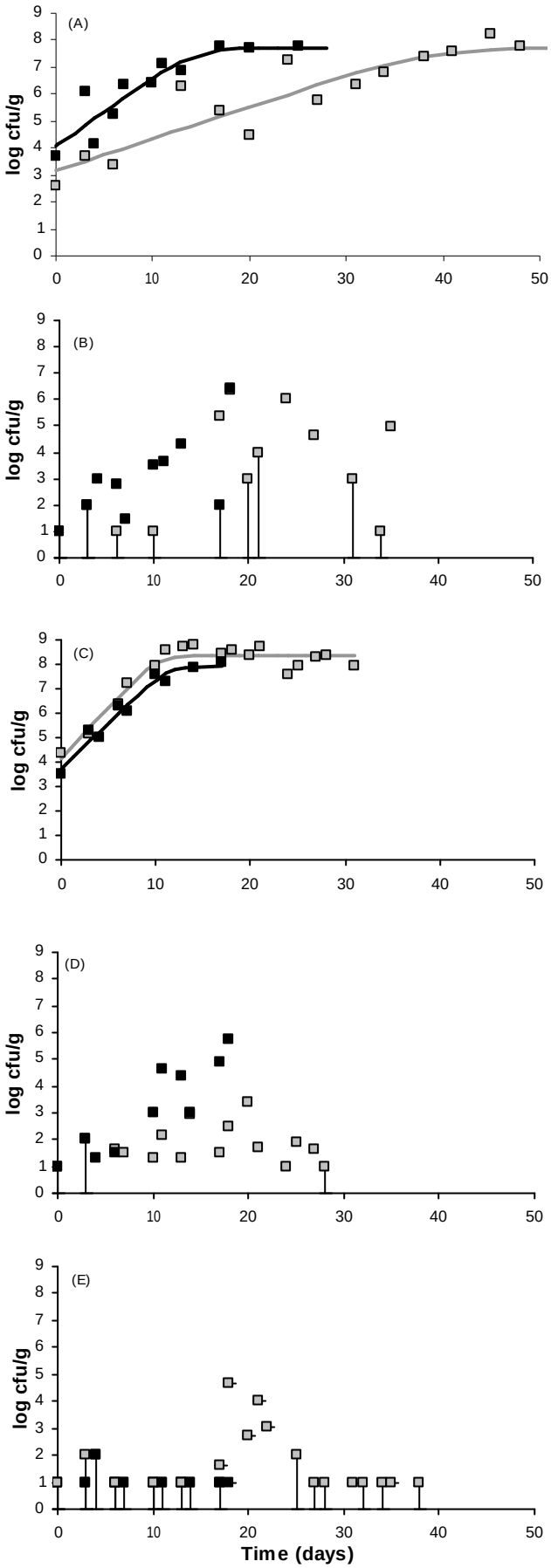


Figure 2



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