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

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19 **Abstract**

20 Salting and smoking are ancient processes for fish preservation. The effects of salt and phenolic
21 smoke compounds on the growth rate of *L. monocytogenes* in cold-smoked salmon were
22 investigated, through physico-chemical analyses, challenge tests on surface of cold-smoked
23 salmon at 4°C and 8°C, and a survey of the literature. Estimated growth rates were compared to
24 predictions of existing secondary models, taking into account the effects of temperature, water
25 phase salt content, phenolic content, and additional factors (e.g. pH, lactate, dissolved CO₂). The
26 secondary model proposed by Devlieghere *et al.* [Food Microbiol. 18 (2001) 53] and modified
27 by Giménez and Dalgaard [J. Appl. Microbiol. 96 (2004) 96] appears appropriate. However,
28 further research is needed to understand all effects affecting growth of *L. monocytogenes* in cold-
29 smoked salmon and to obtain fully validated predictive models for use in quantitative risk
30 assessment.

31

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

34

34 1. Introduction

35 Numerous recent or in-process risk assessments have concerned *Listeria monocytogenes* in
36 sliced and vacuum packed cold-smoked salmon (Buchanan, 1997; Lindqvist and Westöö, 2000;
37 FSANZ, 2002; Beaufort *et al.*, 2002; FDA, 2003; FAO/WHO, 2004). This abundance is clearly
38 justified by the sanitary and economic importance of this issue, but it may also be explained by
39 the relatively good availability of data. *L. monocytogenes* is indeed a well known foodborne
40 pathogen which has been extensively studied since the first major recognised outbreak in the
41 early 1980s (Schlech *et al.*, 1983). Presence and potential growth of this pathogen in cold-
42 smoked salmon has been widely reviewed (Ben Embarek, 1994; Rorvik, 2000; Ross *et al.*,
43 2000). However, as noted in most risk assessment reports, there are still research needs to better
44 characterize the contamination data and to improve and validate the tools of predictive
45 microbiology to predict growth of *L. monocytogenes* in cold-smoked salmon.

46 Predictive microbiology aims to predict microbial behaviour in food over time as a function of
47 different influencing parameters. For a review of such models, see McKellar and Lu (2004).
48 Briefly, primary models describe the evolution of a population of micro-organisms over time
49 under certain conditions whereas secondary models describe how the primary model parameters,
50 e.g. the lag time (*lag*) and the growth rate (μ_{max}), vary with environmental conditions. They are
51 typically based on data generated in liquid laboratory culture media, whereas they aim to predict
52 growth in food products. Validation is then an important issue. Model validation can be defined
53 as demonstrating the accuracy of the model for a specified use. Ross (1996) and Baranyi *et al.*
54 (1999) proposed criteria to measure the performance of a model, i.e. their reliability when
55 compared to independent "real-world" data, obtained in inoculated food products (challenge
56 tests) or even in naturally-contaminated products (storage trials), and not used to generate the

57 model.

58 Dalgaard and Jorgensen (1998) provided an extensive comparison of existing secondary models
59 for *L. monocytogenes*, on the basis of 100 literature challenge tests in different seafood products
60 (including 26 in cold-smoked salmon) and 13 storage trials in cold-smoked salmon. As stated by
61 the authors, one of the limitations was that the inhibiting effect of smoke components was not
62 taken into account, both because no adequate secondary model was available at that time and
63 because the concentration of smoke components was not measured in products used for
64 challenge tests. The antimicrobial activity of smoke is generally attributed to the phenolic
65 fraction, even if no relationship between concentration of phenolic compounds and growth
66 inhibition has clearly been established (Thurette *et al.*, 1998; Niedziela *et al.*, 1998; Suñen *et al.*,
67 2001, 2003; Lebois *et al.*, 2004). Since this study, Augustin and Carlier (2000a, b) have
68 proposed two secondary models taking into account the phenolic content, and Giménez and
69 Dalgaard (2004) have modified two other secondary models to include this phenolic effect.

70 The objective of the present study was (i) to further investigate the physicochemical
71 characteristics of cold-smoked salmon, including the phenolic content, on the basis of a specific
72 survey, and of former similar studies (Leroi *et al.*, 2001; Espe *et al.*, 2004), and (ii) to
73 characterize how they affect growth of *Listeria monocytogenes*, on the basis of specific
74 challenge tests and of literature data (Peterson, *et al.*, 1993; Pelroy, *et al.*, 1994; Rosso *et al.*,
75 1996; Niedziela, *et al.* 1998; Giménez and Dalgaard, 2004; Lakshmanan and Dalgaard, 2004).
76 The four recently proposed secondary models taking into account the concentration of phenolic
77 compounds (Augustin and Carlier, 2000a, b; Giménez and Dalgaard, 2004), and 7 additional
78 secondary models, were compared and evaluated.

79 **2. Materials and methods**

80 *2.1. Physicochemical analyses of French commercial products*

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

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

88 *2.2. Challenge tests (L. monocytogenes) in 5 specific products*

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

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

102 4°C, while non-inoculated off-cuts of the same sub-batch were used for a storage trial at 4°C,
103 whereas the disks and off-cuts of the other sub-batch were used for a challenge test and a storage
104 trial at 8°C. Last, all remaining off-cuts of one batch were pooled and the pool was analysed for
105 pH, salt content, and phenolic compounds according to the procedures detailed above. This
106 pooling was chosen to reach some confidence in estimating the average physicochemical
107 characteristics of each batch, but did not enable us to observe any within-batch physicochemical
108 variability (neither between the sub-batches, nor within a sub-batch).

109 Strain TQA 061, isolated in the laboratory from commercial cold-smoked salmon, and stored at
110 -24°C in a glycerol-containing medium, was used for inoculation of the disks. Prior to challenge
111 testing, the content of one cryotube was thawed, and it was 1/100 diluted in tryptone soya broth
112 (AES, Combourg, France) and cultured 4 days at 10°C. This first preculture in early stationary
113 phase was 1/100 diluted in tryptone soya broth and cultured 3 days at 10°C. This second
114 preculture in early stationary phase was 1/1000 diluted in tryptone salt (AES), to obtain the
115 inoculum suspension, at a level of 2.10^6 cfu per millilitre. A 0.1-ml volume of this inoculation
116 suspension was spread onto each disk, which was then folded, so that the inoculum was
117 sandwiched between the two layers. The folded disks were vacuum-packaged, using a chamber
118 machine Multivac A300/16 (Multivac, Lagny-sur-Marne, France), in polyamide/polyethylene
119 (PA/PE) 30 µm/70µm film (Euralpack, Saint Pierre du Perray, France), with low transmission
120 rates: 30 to 40 $\text{cm}^3 \cdot \text{m}^{-2} \cdot \text{day}^{-1} \cdot \text{bar}^{-1}$ for O₂ (23°C, 75% relative humidity), 90 $\text{cm}^3 \cdot \text{m}^{-2} \cdot \text{day}^{-1} \cdot \text{bar}^{-1}$
121 for CO₂ (23°C, 75% relative humidity), 2.5 $\text{g} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ for H₂O (23°C, 85% relative humidity).
122 For each batch, twenty inoculated 89-mm disks were stored at 4°C±1°C, whereas the twenty
123 other disks were stored at 8°C±1°C, with continuous temperature monitoring. After various
124 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/cm². The theoretical initial contamination,
129 calculated from the contamination level for the inoculation suspension, is 3.5 log cfu/cm², 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 calculate the validation criteria:

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

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

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

$$181 \quad a_w = 1 - 0.0052471 \text{ WPS} - 0.00012206 \text{ WPS}^2 \quad (1)$$

182 When needed in the model and not measured, the water-phase lactate (WPL) level of cold-
183 smoked salmon was assumed to be 90 mM (Tienungoon *et al.*, 2000), which is equivalent to a
184 concentration of sodium lactate (NaL) at 1%. All other concentrations of inhibiting compounds
185 (including dissolved CO₂) were assumed to be null. As Model 7 provides a distribution of
186 predicted growth rates at each temperature, the average of the distribution was chosen for
187 validation.

188 *2.6. Validation criteria*

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

- 190 ■ the accuracy factor, which expresses the accuracy of the model predictions (1 if all
191 predictions are equal to the observations),
- 192 ■ the bias factor, which expresses the overall bias (> 1 for a fail-safe model, < 1 for a fail-
193 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

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

218 3.2. Growth of *L. monocytogenes* in specific products

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

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

236 These growth curves were modelled by a primary model, using the classical least-squares
237 criterion or an alternative robust criterion. Results of the two methods were close (Table 2) and
238 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

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

270 For these two batches, and especially for batch A, the population levels reached by the food flora
271 in storage trials is close or even higher than the contamination levels of *L. monocytogenes* in
272 challenge tests. Then, it is possible that, in these two batches, the observed growth of
273 *L. monocytogenes* had been influenced by the simultaneous growth of a non-neglectable or even
274 predominant background flora. It has often been observed that the major interaction observed in
275 cold-smoked salmon between the background flora (among which the lactic acid flora tends to
276 be predominant) and *L. monocytogenes* is a competition, the so-called Jameson effect, with a
277 simultaneous deceleration of all populations (see Buchanan *et al.*, 1997, 1999; Dalgaard and
278 Jorgensen, 1998; Ross *et al.*, 2000; Cornu, 2001; FAO/WHO, 2004; Giménez and Dalgaard,
279 2004; Nilsson *et al.*, 2005). Even if the data are not appropriate to detect such an effect, the
280 deceleration of *L. monocytogenes* at a relatively low level (ca. 10⁶ cfu/cm²), in batch A at 8°C,
281 after ca. 20 days, could be explained by the simultaneous deceleration of the food flora
282 (observed in storage trials after ca. 17 days at 8°C).

283 This Jameson effect only impacts the maximum population density of *L. monocytogenes* and not
284 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

308 significantly non null, but its biological significance can be questioned. Indeed, with a null lag
 309 time, the estimated growth rate is 0.92 day^{-1} , which appears more consistent with the predictions.
 310 This example is an indication that the estimation procedure of the growth parameters is much
 311 more complex in the case of challenge tests, than in the case of curves in broth.
 312 For the sake of comparison, models 1, 3 and 4 were rewritten into a unified five-parameter
 313 equation:

$$314 \quad \mu = \mu_{\text{ref}} \cdot \frac{(T - T_{\text{min}})^2}{(T_{\text{ref}} - T_{\text{min}})^2} \cdot \frac{(WPS - WPS_{\text{max}}) + 0.02326 (WPS^2 - WPS_{\text{max}}^2)}{(WPS_{\text{ref}} - WPS_{\text{max}}) + 0.02326 (WPS_{\text{ref}}^2 - WPS_{\text{max}}^2)} \cdot \frac{(P_{\text{max}} - P)^k}{(P_{\text{max}} - P_{\text{ref}})^k} \quad (2)$$

315 where T_{min} is the minimal temperature, WPS_{max} the MIC-value for WPS, calculated from the
 316 minimal a_w of each model, using Equation (1), P_{max} the phenolic MIC-value, k equals 1 or 2, and
 317 μ_{ref} the predicted growth rate for a reference cold-smoked salmon at a reference temperature, *i.e.*
 318 the prediction of the model for the following conditions: $T = T_{\text{ref}} = 5^\circ\text{C}$; $\text{pH} = 6.20$; $WPS_{\text{ref}} =$
 319 5.0% ; $P = P_{\text{ref}} = 1.0 \text{ mg}/100\text{g} = 10 \text{ ppm}$; $WPL = 8000 \text{ ppm} = 90 \text{ mM}$, corresponding to $\text{NaL} =$
 320 1% ; $\text{CO}_{2\text{diss}} = 0 \text{ ppm}$.

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

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

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

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

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

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

350 Thus, the described between-product physicochemical variability does not appear sufficient to
351 fully explain the between-curve variability of growth rates. Our description of the between-
352 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 an 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

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

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

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

390 **4. Conclusion**

391 Physiochemical characteristics of cold-smoked salmon, especially the contents in salt and
392 phenolic compounds, affect growth rates of *L. monocytogenes*. Secondary models can be used to
393 model these effects and, among the four tested models, the secondary model proposed by
394 Devlieghere *et al.* (2001) and modified by Giménez and Dalgaard (2004) appeared the most
395 appropriate one. However, it was obvious that the studied factors, including the phenolic content,
396 were not sufficient to describe the whole variability of the behaviour of *L. monocytogenes* in
397 cold-smoked salmon. Additional sources of uncertainty and variability affecting the growth rate
398 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**

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522 **Figure 1.** Growth curves of *L. monocytogenes* on cold-smoked salmon (log cfu/cm²), 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
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531 primary model without lag (grey solid line: 4°C, black solid line: 8°C).

532

533 **List of Tables**

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

539
540 **Table 2:** Estimated and predicted growth rates (day^{-1}) of *L. monocytogenes* on cold-smoked
541 salmon at 4°C and 8°C. Estimations were obtained fitting each growth curve using the chosen
542 model (see Figure 1) and non-linear least-squares and robust regression (Miconnet *et al.*, in
543 press). Predictions were obtained using four secondary models, and the physico-chemical
544 characteristics of each product (see Table 1). Model 1: Augustin and Carlier (2000a). Model
545 2: Augustin and Carlier (2000b). Model 3: Ross (FAO/WHO, 2004), modified by Giménez
546 and Dalgaard (2004). Model 4: Devlieghere et al (2001), modified by Giménez and Dalgaard
547 (2004).

548
549 **Table 3.** Parameters of three models in a unified and simplified equation (see Equation (2) in
550 the text). Model 1: Augustin and Carlier (2000a). Model 3: Ross (FAO/WHO, 2004),
551 modified by Giménez and Dalgaard (2004). Model 4: Devlieghere et al (2001), modified by
552 Giménez and Dalgaard (2004).

553
554 **Table 4.** Validation criteria of models 1 to 4 based on 41 growth rates of *L. monocytogenes*:
555 10 challenge tests on cold-smoked salmon in the present study (see Figure 1 and Table 2, the
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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).

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Table 1.

	pH	Salt content (g/100g)	WPS (g/100ml)	Water content	P = phenolic content (mg/100g)
<i>40 commercial French products (present survey):</i>					
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]
<i>13 commercial French products (Leroi et al., 2001):</i>					
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]
<i>48 commercial French products (Espe et al., 2001):</i>					
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]
<i>5 specific batches, used for challenge tests:</i>					
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 Batch, Temp.	Estimated growth rates (day ⁻¹)		Predicted growth rates (day ⁻¹)			
	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
<i>10 growth rates of the present study</i>											
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
<i>9 growth rates in cold-smoked salmon (literature)</i>											
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
<i>10+9=19 growth rates in cold-salmon products (all sources)</i>											
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
<i>22 growth rates in salted (non-smoked) salmon (literature)</i>											
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

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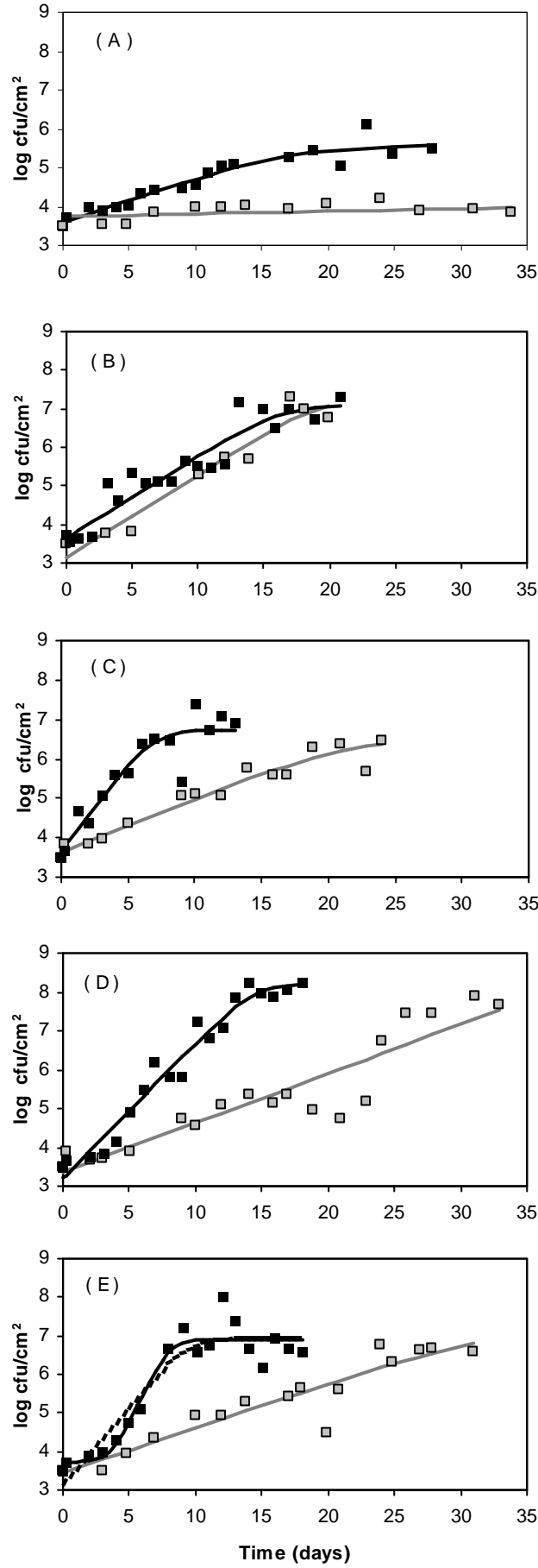


Figure 2

