Statistical Modelling of Cold-Smoked Salmon Temperature Profiles for Risk Assessment of *Listeria monocytogenes*

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Abstract

In predictive microbiology, the bacterial growth is expressed as a function of pH, water activity and temperature. The temperature is usually assumed to be constant. Nevertheless, in the cold chain, temperature fluctuations are observed. This paper proposes a statistical modelling for the temperature evolution of a cold-smoked salmon, in the framework of a risk assessment of *L. monocytogenes*.

INTRODUCTION

Consumer food safety depends on temperature control throughout all stages in the cold chain (Gac and Durand, 2001; Willocx et al., 1993). The preservation of food-stuffs at low temperature decelerates the bacterial growth. In predictive microbiology, the bacterial growth is expressed as a function of pH, water activity and temperature. The temperature is usually assumed to be constant. But in practice, temperature fluctuations are observed in the cold chain. The originality of this work is to take into account these temperature fluctuations in order to evaluate the bacterial growth.

This study is part of a joint project between AFSSA and CEMAGREF, whose aim is to determine the exposure to the hazard *Listeria monocytogenes* using bacterial growth models (Cornu and Beaufort, 2005). A statistical modelling for the temperature evolution of a cold-smoked salmon is proposed.

MATERIAL AND METHODS

Data

The cold chain contains different stages, such as transport (T), storage (E), platform (P), cold room in store (C), retail display cabinet (M), transport after purchase (V), domestic refrigerator (R) and domestic preservation at ambient temperature (D). Some stages occur several times during product shelf life.

In our experiments (Table 1), the product temperature was recorded at each step of the cold chain. In fact, it was not possible, for technical reasons, to follow all the stages of a same product from the factory to the consumption. The data loggers are programmed to record the product temperature every 15 minutes.

Predictive Microbiology Models

In predictive microbiology, the growth of one *L. monocytogenes* strain in a cold-smoked salmon is described by an exponential primary model (Equation 1) (Van Gerwen and Zwietering, 1998). The lag phase was neglected (sail-safe assumption). The stationary phase is not taken into account here but is modeled in Cornu and Beaufort (2005). We chose as secondary model the square-root model (Equation (2)) to express the influence of temperature on the growth parameters (Van Gerwen and Zwietering, 1998).

The two equations can be written as follows:

$$\ln(N_{t_k}) = \ln(N_{t_{k-1}}) + \mu(\theta_{ij(k)})\Delta t_k \quad (1)$$

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$$\mu(\theta_{ij(k)}) = b^2(\theta_{ij(k)} - \theta_{min})^2 \quad (2)$$

where:

- i is one of the 8 stages along the cold chain with i=T, E, P, C, M, V, R, D,
- j is one of the stages of the same kind, j=1, …, p_i,
- k is the recorded temperature index in the stage i with k=1, …, n_{ij},
- $\theta_{ij(k)}$ is the recorded temperature at time t_k (°C),
- Δt_k is the time interval with $\Delta t_k = t_k - t_{k-1}$. In our experiments, Δt_k is constant and $\Delta t_k = \Delta t = 15$min,
- N_{t_k} is the bacterial population at time t_k with N_0 the initial bacterial population (cfu.ml^{-1}),
- μ is the bacterial growth rate with μ positive (h^{-1}),
- b is a parameter of the square root model, characterising the couple considered by the bacterial strain and the food product (°C^{-1}.h$^{-0.5}$),
- θ_{min} is the theoretical minimal temperature of growth for the considered bacterial strain (°C).

After incrementation of Equation (1) during the simulated duration of considered stage i, subdivided into n_{ij} intervals, and after having combined Equation (1) and Equation (2), we deduce Equation (3):

$$\ln(N) = \ln(N_0) + b^2 \left[\Delta t \sum_{k=1}^{n_{ij}} \theta_{ij(k)}^2 - 2\theta_{min} \Delta t \sum_{k=1}^{n_{ij}} \theta_{ij(k)} + \theta_{min}^2 n_{ij} \Delta t \right] \quad (3)$$

where N is the bacterial population at the end of the stage and N_0 the bacterial population at the beginning of the stage. Equation (3) can be rewritten in a simplified way (Equation 4), using d_{ij} the duration of a stage or residence time in this stage, $\hat{m}_{ij}$ the average temperature during the time interval d_{ij} and $\hat{\sigma}_{ij}^2$ the temperature variance (biased estimate), defined in Equation (5):

$$\begin{aligned} \ln(N) &= \ln(N_0) + b^2 \left[d_{ij}(\hat{\sigma}_{ij}^2 + \hat{m}_{ij}^2) - 2\theta_{min} \hat{m}_{ij} d_{ij} + \theta_{min}^2 d_{ij} \right] \\ &= \ln(N_0) + b^2 d_{ij} \left[\hat{\sigma}_{ij}^2 + (\hat{m}_{ij} - \theta_{min})^2 \right] \end{aligned} \quad (4)$$

$$\begin{cases} d_{ij} = \sum_{k=1}^{n_{ij}} \Delta t = n_{ij} \Delta t \\ \hat{m}_{ij} = \dfrac{\sum_{k=1}^{n_{ij}} \theta_{ij(k)}}{n_{ij}} \Rightarrow \sum_{k=1}^{n_{ij}} \theta_{ij(k)} = \hat{m}_{ij} n_{ij} = \hat{m}_{ij} \dfrac{d_{ij}}{\Delta t} \\ \hat{\sigma}_{ij}^2 = \dfrac{\sum_{k=1}^{n_{ij}} \theta_{ij(k)}^2}{n_{ij}} - \hat{m}_{ij}^2 \Rightarrow \sum_{k=1}^{n_{ij}} \theta_{ij(k)}^2 = n_{ij}(\hat{\sigma}_{ij}^2 + \hat{m}_{ij}^2) = \dfrac{d_{ij}}{\Delta t}(\hat{\sigma}_{ij}^2 + \hat{m}_{ij}^2) \end{cases} \quad (5)$$

Thus, without any loss of information, the chosen predictive microbiology models rely on only three time-temperature variables (d_{ij}, $\hat{m}_{ij}$ and $\hat{\sigma}_{ij}^2$) and three microbial variables (n_0, b^2 and T_{min}). In order to assess the bacterial number, this paper focuses on the three time-temperature variables on which we fit a statistical distribution.

Statistical Modelling

Variability distributions were obtained for each variable, d_{ij}, $\hat{m}_{ij}$ and $\hat{\sigma}_{ij}^2$ in each stage. The variable d_{ij} is defined as the duration between the beginning and the end of the stage. According to Vose (2000), it is described by an exponential distribution $\xi(\alpha)$ with density:

$$f_{d_{ij}}(t) = \frac{1}{\alpha} e^{-\frac{t}{\alpha}} \quad (6)$$

where parameter α is estimated from its average value. The exponential distribution for duration is applied to all stages of the cold chain. The following parts concern the two other statistical variables: $\hat{m}_{ij}$ and $\hat{\sigma}^2_{ij}$.

Let us consider a sample constituted by n_{ij} temperatures $\theta_{ij(1)},...,\theta_{ij(k)},...,\theta_{ij(n_{ij})}$, for stage *i* of the cold chain with i=T, E, P, C, M, V, R, D, j=1, …, p_i and k=1, …, n_{ij}. When applying the Central Limit Theorem to sample $(\theta_{ij(1)},...,\theta_{ij(k)},...,\theta_{ij(n_{ij})})$, two main assumptions are used:

- Hypothesis H_1: the n_{ij} random variables $\theta_{ij(1)},...,\theta_{ij(k)},...,\theta_{ij(n_{ij})}$ are independent,
- Hypothesis H_2: the n_{ij} random variables $\theta_{ij(1)},...,\theta_{ij(k)},...,\theta_{ij(n_{ij})}$ are identically distributed

i.e.

$$\begin{cases} E[\theta_{ij(1)}] = ... = E[\theta_{ij(k)})] = ... = E[\theta_{ij(n_{ij})})] = m_{ij} \\ \text{var}[\theta_{ij(1)}] = ... = \text{var}[\theta_{ij(k)})] = ... = \text{var}[\theta_{ij(n_{ij})})] = \sigma^2_{ij} \end{cases} \quad (7)$$

Then, the variable "average temperature" computed on the sample of temperatures with size n_{ij} follows the normal distribution $N(m_{ij}, \sigma^2_{ij}/n_{ij})$ with parameters estimation using the following expressions:

$$\begin{cases} \hat{m}_{ij} = \dfrac{1}{n_{ij}} \sum_{k=1}^{n_{ij}} \theta_{ij(k)} \\ \hat{\sigma}^2_{ij} = \dfrac{1}{n_{ij}} \sum_{k=1}^{n_{ij}} (\theta_{ij(k)} - \hat{m}_{ij})^2 \end{cases} \quad (8)$$

For a stage type, we observed several average temperatures. So, we can determine a variability distribution for $\hat{m}_{ij}$ (Vose, 2000). Thus, $\hat{m}_{ij}$ follows the normal distribution $N(m_i, \sigma^2_i)$ with parameters estimation using the following expressions:

$$\begin{cases} \hat{m}_i = \dfrac{1}{p_i} \sum_{j=1}^{p_i} \hat{m}_{ij} \\ \hat{\sigma}^2_i = \dfrac{1}{p_i - 1} \sum_{j=1}^{p_i} (\hat{m}_{ij} - \hat{m}_i)^2 \end{cases} \quad (9)$$

The normal distribution for average temperature is applied to all stages of the cold chain. When the theoretical variance of the biased estimator $\hat{\sigma}^2_{ij}$ is different from zero, the adjustment factor follows a Gamma distribution according to the following statistical result:

$$\frac{n_{ij}}{\sigma^2_{ij}} \hat{\sigma}^2_{ij} \equiv \chi^2(n_{ij} - 1) \Rightarrow \hat{\sigma}^2_{ij} \equiv \Gamma\left(\frac{n_{ij} - 1}{2}, \frac{n_{ij}}{2\sigma^2_{ij}}\right) \text{ with } \hat{\sigma}^2_{ij} = \frac{1}{n_{ij}} \sum_{k=1}^{n_{ij}} (\theta_{ij(k)} - \hat{m}_{ij})^2 \quad (10)$$

Thus, chosen statistical distributions are exponential for d_{ij}, normal for $\hat{m}_{ij}$ and gamma for $\hat{\sigma}^2_{ij}$ (Nauta et al., 2003).

RESULTS AND DISCUSSION

To assess the growth of *Listeria monocytogenes* in cold-smoked salmon along the cold chain, we simulate different logistical chains. Table 2 presents these different logistical chains reproduced from experiments and expertise. The percent of "first stages" is based on our expertise, the percent of "last stages" on our experiments. Then, we cross a line (for example MVD) and a column (for example TPT) to compose a predicted chain. It can be observed on Table 2 that there is 1% of chains with type TPTMVD; this percent results from the product of 38.1% (total TPT) and 2.6% (total MVD).

To reproduce a chain (for example TPTMVD) in the bacterial growth model, we randomly sample the three chosen statistical variables (duration, average temperature and variance) for each stage. The duration is sampled from an exponential distribution (Table 3), fitted to the observed durations (Fig. 3). The average temperature is sampled from a Normal distribution (Tables 4 and 5) fitted to the observed data (Fig. 3). The temperature variance is either sampled from a Gamma distribution (Table 4) fitted to the observed positive variances (Fig. 2) or equal to zero. These three statistical variables are choosen, in such a way that all the necessary information to model microbial growth was available. They are used as inputs in the predictive microbiology model in order to assess the risk of *Listeria monocytogenes* in cold-smoked salmon.

CONCLUSIONS

The statistical approach proposed in this paper can be a way to represent the time-varying temperatures of a product in the cold chain. The obtained statistical distributions can be used as input variables for a computational food risk model, based on Monte Carlo simulations, and an example of such a model is presented in this conference (Cornu and Beaufort, 2005). Nevertheless, the correlation problem between the random variables within a stage or between stages has to be taken into account.

ACKNOWLEDGEMENTS

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Tables

Table 1. Number of stages where the temperature of cold-smoked salmon has been recorded.

STAGE i	Sample size: p_i
Refrigerated transport (T)	181
Entrepot/Storage (E)	12
Platform (P)	140
Cold room (C)	49
Market / Retail display cabinet (M)	67
Vehicle / Transport after purchase (V)	77
Domestic refrigerator (R)	68
Domestic preservation at ambient temperature (D)	20

Table 2. Probability of the predicted logistical chain types.

first stages / last stages	1T (T)	2T+1P (TPT)	3T+1E+1P (TETPT)	3T+2P (TPTPT)	4T (TPTPTPT)	Total
MV	0.2%	2.8%	2.6%	1.4%	0.45%	*7.5%*
CMV	0.004%	0.06%	0.06%	0.03%	0.01%	*0.2%*
M V D	0.1%	1.0%	0.9%	0.4%	0.2%	*2.6%*
M V R	1.2%	19.8%	18.1%	9.1%	3.7%	*51.8%*
C M V R	0.3%	5.1%	4.7%	2.3%	1.0%	*13.4%*
M V R D	0.6%	8.5%	7.8%	3.9%	1.6%	*22.4%*
C M V R D	0.05%	0.8%	0.7%	0.3%	0.1%	*2.0%*
Total	*2.4%*	*38.1%*	*34.9%*	*17.5%*	*7.1%*	*100.00%*

Table 3. Adjustment of the duration by an exponential distribution.

Cold chain stage	Variable Duration
Refrigerated transport (T)	ξ(0.076 day)
Storage (E)	Fixed : 0.7 day
Platform (P)	ξ(0.49 day)
Cold room in store (C)	ξ(0.96 day)
Retail display cabinet (M)	ξ(4.6 days)
Vehicle / Transport after purchase (V)	ξ(0.046 day)
Domestic refrigerator (R)	ξ(4.8 days)
Domestic preservation at ambient temperature (D)	ξ(0.3 day)

Table 4. Adjustment of the average temperature and temperature variance by statistical distributions (with $s_{ij}^2 \neq 0$).

STAGE / VARIABLE	% of stages with $s_{ij}^2 \neq 0$	Average of temperature with $s_{ij}^2 \neq 0$	Variance of temperature
Refrigerated transport (T)	46%	N(3.6;2.7)	Γ(0.6;2.3)
Storage (E)	100%	N(3.6;2.8)	Γ(2.2; 0.7)
Platform (P)	84%	N(2.9;2.9)	Γ(0.5;0.8)
Cold room in store (C)	71%	N(4.4;1.9)	Γ(0.7;1.1)
Retail display cabinet (M)	100%	N(5.6;2.2)	Γ(1;0.9)
Vehicle / Transport after purchase (V)	88%	N(13;3.6)	Γ(0.6;0.1)
Domestic refrigerator (R)	100%	N(7;3)	Γ(1.3;0.6)
Domestic preservation at ambient temperature (D)	85%	N(17.9;5.1)	Γ(0.6;0.1)

Table 5. Adjustment of the average temperature and temperature variance by statistical distributions (with $s_{ij}^2 = 0$).

STAGE / VARIABLE	% of stages with $s_{ij}^2 = 0$	Average Temperature with $s_{ij}^2 = 0$	Variance of temperature
Refrigerated transport (T)	54%	N(2.7;3.4)	0
Platform (P)	16%	N(0.1;3.8)	0
Cold room in store (C)	29%	N(2.7;1.5)	0
Vehicle / Transport after purchase (V)	12%	N(10.4;1.5)	0
Domestic preservation at ambient temperature (D)	15%	N(13.2;5.2)	0

Figures

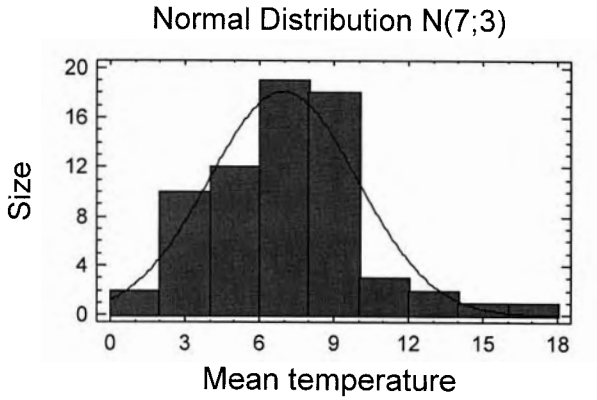

Fig. 1. Adjustment of average temperature in domestic refrigerator (R) by a normal distribution.

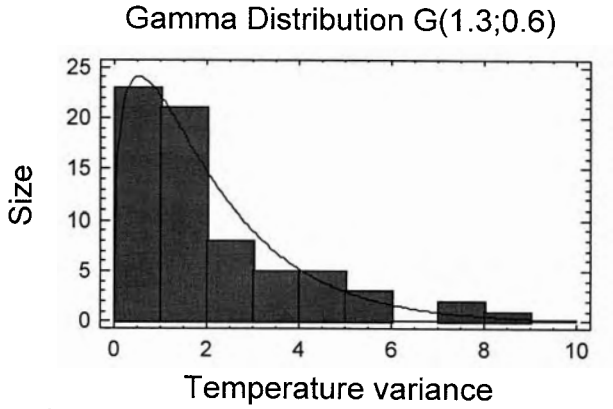

Fig. 2. Adjustment of temperature variance in domestic refrigerator (R) by a Gamma distribution.

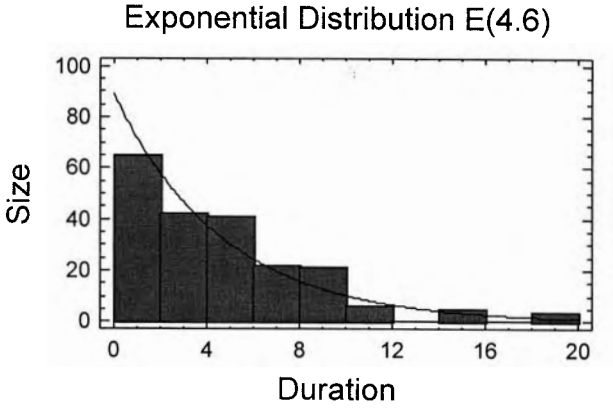

Fig. 3. Adjustment of duration in retail display cabinet (M) by an exponential distribution.

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Technologie de préparation du saumon fumé : incidence du froid sur le développement microbien

d'Hélène Bergis*

La préparation du saumon fumé se fait suivant des technologies qui diffèrent notamment par les températures utilisées. Cet article présente des exemples de profils thermiques et étudie leurs conséquences sur le temps de latence de Listeria monocytogenes.

En quelques années la consommation de saumon fumé a beaucoup évolué. Ce produit, considéré il y a encore quelques temps comme un aliment de luxe, a tendance à être consommé plus couramment.

Données relatives à la consommation du saumon fumé en France

Entre 1997 et 1998, la consommation de saumon fumé à domicile a fortement augmenté, atteignant 13 287 tonnes en 1998, ce qui représentait une croissance de 7 % en volume. Il semble que les années 2000-2001 ont été moins favorables, une baisse de 6 % ayant été enregistrée durant cette période [1]. Cette diminution est certainement à mettre en relation avec la hausse du prix du saumon fumé (hausse de 6 % du prix moyen), due à une augmentation du prix d'achat à l'importation de la matière première.

L'exploitation des données Secodip de 1997 sur la consommation des ménages en France (données d'achat portant sur 5 348 foyers durant une période

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Résumé

La technologie de préparation du saumon fumé a été appréhendée dans différents ateliers de production. Au cours de ces visites, les profils thermiques à chaque étape du process de préparation (salage, fumage, maturation, tranchage, conditionnement) ont été établis. Certaines de ces étapes, réalisées à des températures négatives, peuvent induire un stress thermique sur la microflore présente dans le saumon. L'influence de ce stress sur la croissance de *Listeria monocytogenes*, introduite artificiellement dans la matrice du saumon fumé, a été étudiée et les temps de latence ont été déterminés. Au vu de ces premiers résultats, il semblerait que les chocs thermiques, associés aux différentes techniques de préparation utilisées, n'aient que peu de conséquences sur la durée de latence de ce micro-organisme dans l'aliment.

Abstract

Technology of preparation of smoked salmon was observed in different companies. During these visits, thermal history at each step of the process (salting, smoking, maturation, slicing, packaging) were studied. Some of these steps, made at freezing temperatures, can induce a cold shock on the microflora present in salmon. The effect of this stress on the growth of Listeria monocytogenes *artificially introduce in the smoked salmon was studied and the lag time determined. According to the first results obtained in this study, it seems that cold shocks linked to the different technology of preparation have only few consequences on the lag time duration of this micro-organism in food.*

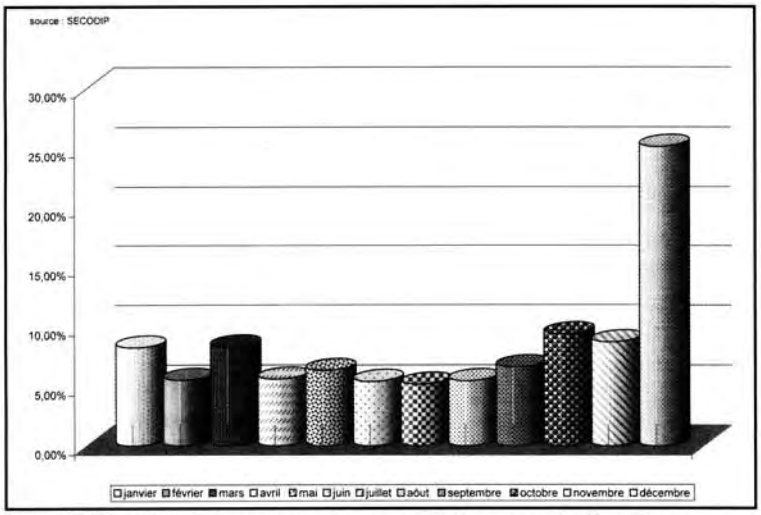

Figure 1. *Répartition de la consommation annuelle de saumon fumé en France.*

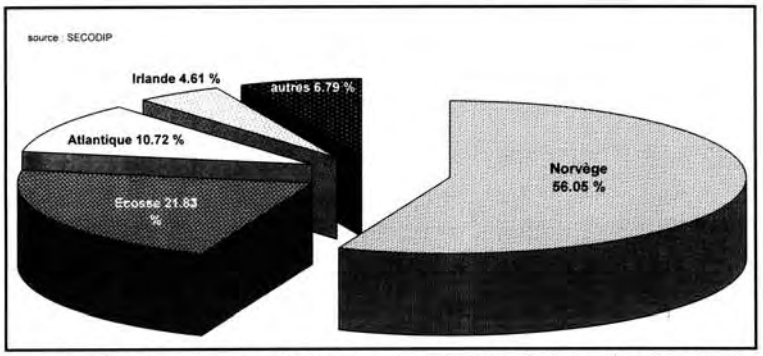

Figure 2. *Répartition des actes d'achat du saumon fumé en France en fonction de sa provenance.*

d'un an) fait apparaître une augmentation notable de la consommation de saumon fumé au mois de décembre (figure 1). Ce produit reste donc toujours étroitement associé aux périodes festives de fin d'année, mais se consomme régulièrement tout au long de l'année (consommation moyenne de 6,8 % sur les onze premiers mois). La ventilation des actes d'achat en fonction de la provenance des saumons est présentée figure 2. Il apparaît clairement que le saumon provenant de Norvège est le plus consommé. Il représente à lui seul 56 % des achats de saumon fumé en France.

Les producteurs de saumon fumé, soucieux de répondre à l'attente des consommateurs, tant sur la diversité des produits proposés, que sur leur qualité (qualité organoleptique et microbiologique), améliorent et perfectionnent sans cesse leur technologie de préparation.

Technologie de préparation du saumon fumé

Les industriels français du saumon fumé s'approvisionnent en saumons frais d'élevage auprès des trois prin-

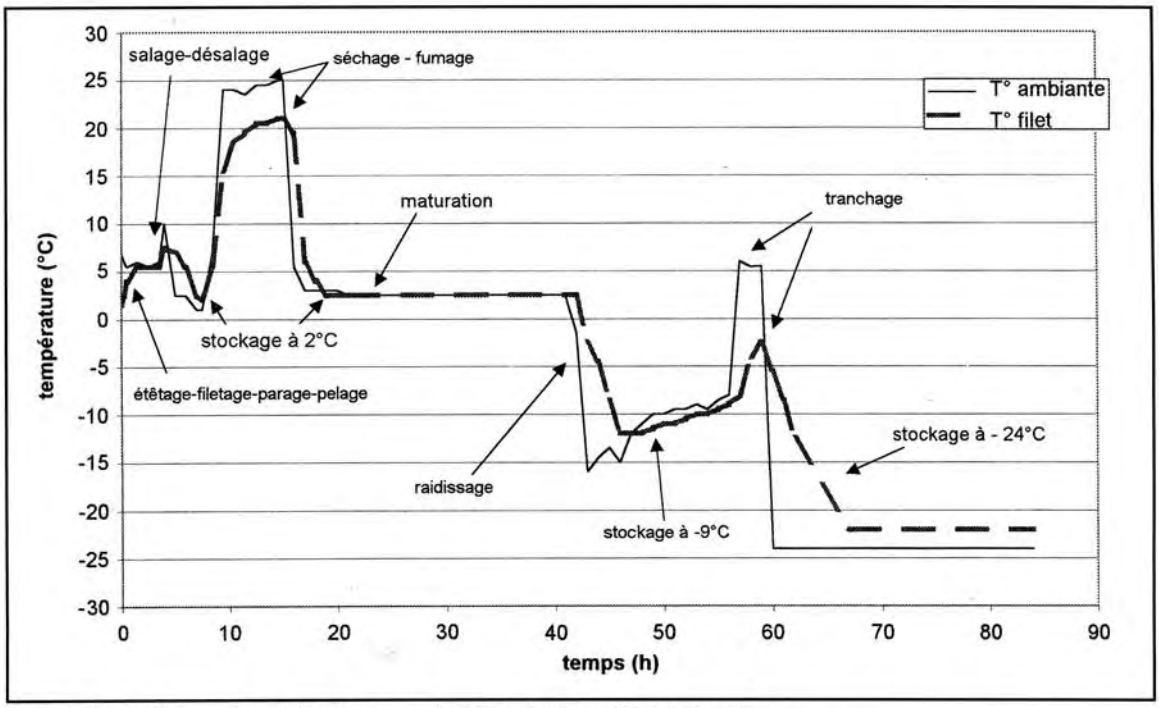

Figure 3. *Un profil thermique de filet de saumon fumé lors d'un procédé de préparation.*

cipaux pays européens producteurs (Norvège, Ecosse et Irlande).

Pour garantir au mieux la qualité bactériologique de la matière première, ces derniers sélectionnent leurs fournisseurs et tentent ainsi, par cette première mesure, de prévenir la contamination par *Listeria monocytogenes*, qui reste, malgré les mesures d'hygiènes prises dans les ateliers de fabrication, « l'épée de Damoclès » de la profession.

Afin de mieux connaître les process de préparation utilisés dans le domaine industriel, d'avoir une idée de la variabilité de ces conditions de process et par là même de caractériser les profils thermiques du saumon fumé, nous avons suivi la préparation de ce produit dans différentes entreprises.

Pour réaliser ce suivi, des enregistreurs de température programmés toutes les 5 minutes ont été utilisés. Ils ont été insérés dans la chair des filets de saumon pour mesurer la température à cœur, et placés dans l'air ambiant pour connaître les conditions environnantes. La figure 3 représente un des profils thermiques enregistrés au cours d'un process de préparation dans une grande entreprise. Sur ce graphique figure la température des filets de saumon, mais également la température ambiante des différents ateliers ou chambres froides dans lesquels transitent les filets de saumon.

Photo 1 : *enceintes pour le séchage-fumage des filets de saumon.*

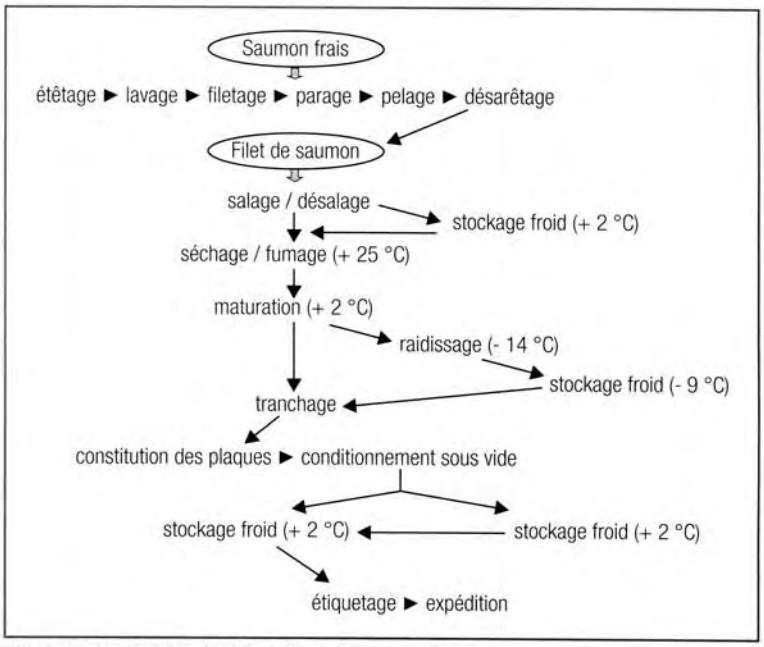

Figure 4. *Diagramme de préparation du saumon fumé*

Profils thermiques enregistrés au cours du process

À leur arrivée dans les ateliers de transformation, les saumons frais éviscérés, conservés dans de la glace en paillette, sont stockés dans leur conditionnement d'origine (caisse polystyrène) en chambre froide, entre 0°C et 3°C.

Les transformateurs de saumon travaillant en flux tendu, les poissons frais se retrouvent rapidement sur la chaîne de fabrication.

Ainsi, pour la gamme de saumon « Label Rouge », les industriels sont tenus de ne pas dépasser six jours entre la date d'abattage et de filetage.

Commence alors toute une série d'opérations : étêtage, filetage, parage, pelage et « désarêtage » qui sont réalisées en chaîne, manuellement ou mécaniquement (voir figure 4).

Le temps qui s'écoule entre le moment où le poisson est décaissé et celui où les filets de saumon sont prêts pour l'étape de salage est très court, de l'ordre de 30 minutes.

Ces opérations n'engendrent donc pas d'élévation importante de la température du saumon, d'autant plus qu'elles sont effectuées dans des ateliers à température dirigée, comprise entre 6° et 12°C. La température des saumons au cours des ces premières opérations oscille entre 0°C et 2°C.

Les filets ainsi obtenus sont alors salés. Plusieurs techniques de salage [2] peuvent être utilisées : le salage au sel sec, réalisé soit manuellement, soit mécaniquement par aspersion d'un brouillard de sel ; le salage par injection de saumure ou bien encore un salage mixte alliant à la fois l'usage du sel sec et de la saumure.

La durée de salage est fonction du calibre du saumon mais également de sa teneur en matière grasse. Les durées de salage enregistrées sont donc très variables et s'échelonnent de 4 h à 16 h. Quant à la température des filets, elle varie entre 2°C et 9°C.

À cette opération de « salage-désalage », succède l'étape de « séchage-fumage » (voir figures 3 et 4). En France le fumage est réalisé à 25°C, c'est ce que l'on appelle le fumage à froid.

Dans les pays d'Amérique du nord (Canada et USA), il se pratique à chaud, à des températures supérieures à 60°C [3], températures commençant à avoir un effet assainissant sur le produit.

Le « séchage-fumage » (voir photo 1) réalisé dans des enceintes à 25°C, dure de 6 heures à 10 heures et la température des filets atteint 20°C à 23,5°C au moment du fumage. Là encore, la durée du traitement est très hétérogène et dépend notamment de la taille des filets.

Une étape de maturation, permettant au sel et à la fumée de se répartir de façon homogène dans les filets, est ensuite réalisée à basse température.

Les durées, plus ou moins longues, de stockage en chambre froide à 2°C engendrent des différences notables sur la température des filets à la fin de ce stockage.

Ainsi, à l'issue d'une période de stockage minimale de 4 à 6 heures, la température des filets est de l'ordre de 8°C. Pour des filets stockés pendant 24 à 48 heures, leur température atteint 2°C, température de la chambre froide.

Après cette période transitoire, les filets de saumons sont tranchés. En fonction du matériel de tranchage utilisé, les filets de saumon peuvent subir une étape supplémentaire dite de raidissage.

Ce raidissage peut être réalisé, soit par froid mécanique, avec un entreposage des filets pendant 4 heures dans une enceinte à - 14°C, soit par froid cryogénique où les filets sont très rapidement refroidis à des températures de - 7 à - 8°C. Quelle que soit la technique de raidissage utilisée, les filets de saumon sont ensuite stockés en chambre froide négative à - 9°C en attendant d'être tranchés. Dans les entreprises de très grande taille, où les cadences de production doivent répondre à une forte demande, les filets de saumon raidis passent sur des lignes de tranchage très sophistiquées qui effectuent, outre le tranchage, un calibrage des tranches et une reconstitution automatique des plaques (voir photo 2).

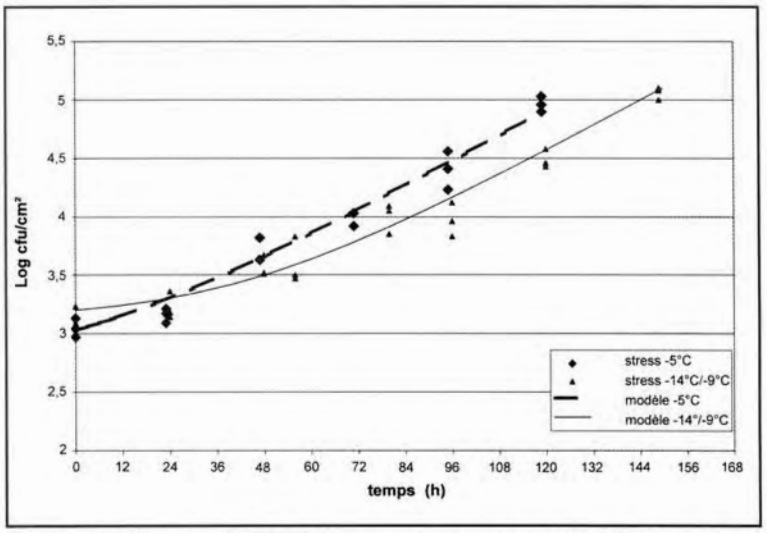

Figure 5. *Courbes de croissance de Listeria monocytogenes à 8°C après un stress à - 5°C et un stress à - 14°C/- 9°C pendant une semaine.*

Photo 2 : *Lignes de tranchage pour le saumon.*

Cette technologie permet d'obtenir, rapidement et sans l'intervention d'opérateurs, des plaques de saumon ayant les caractéristiques requises, à savoir poids fixe et nombre de tranches fixe.

En dehors de la technique du raidissage, deux autres pratiques peuvent être utilisées. Soit les filets de saumon sortent de la chambre de maturation et sont directement tranchés, soit, pratique plus marginale, le tranchage est réalisé après stockage des filets en chambre froide à - 5°C (température proche du point de congélation du saumon fumé qui est de l'ordre de - 3°C).

Les plaques de saumon ainsi constituées sont ensuite conditionnées sous vide et conservées en chambre froide à températures positive (environ 2°C) ou négative (environ - 24°C), avant expédition.

Le process de préparation du saumon fumé est donc composé d'une série de traitements, où chaque étape peut être à l'origine d'un stress pour la microflore présente dans le saumon [4], freinant ainsi l'altération du produit (stress froid, stress NaCl, stress phénol, stress anaérobiose).

Nous nous sommes particulièrement intéressés à l'influence du stress thermique de ce process sur le temps de latence de *Listeria monocytogenes*, micro-organisme pathogène poten-

tiellement présent dans cette matrice et pouvant se développer à basse température.

Stress froid et temps de latence

Ayant caractérisé ces profils thermiques sur site, nous avons donc envisagé de reproduire deux stress thermiques d'intensités variables parmi ceux observés, et de quantifier leur incidence sur le temps de latence de *Listeria monocytogenes*.

Actuellement, il semble difficile de prédire le temps de latence des micro-organismes, celui-ci dépendant notamment de l'état physiologique de la bactérie mais également des conditions environnementales de croissance [5] (température, teneur en NaCl, taux de phénol, pH, conditions d'anaérobioses).

Les deux stress thermiques reproduits sont les suivants : une conservation des échantillons de saumon à - 5°C pendant une semaine pour le premier stress et une conservation à - 14°C pendant 4 heures, suivi d'une conservation à - 9°C pendant une semaine pour le second stress.

Ces profils thermiques sont appliqués à des échantillons de saumon fumé contaminés artificiellement en surface par une souche de *Listeria monocytogenes* provenant de saumon fumé, cultivée à 10°C et se trouvant en phase stationnaire de croissance.

Ces échantillons ainsi contaminés à environ 10^3 cfu/g. sont conditionnés sous vide et stockés aux températures négatives précisées ci-dessus.

La période de stockage terminée, ces échantillons sont ensuite transférés à 8°C et une courbe de croissance de *Listeria monocytogenes* est établie pour chacun des stress thermiques (voir figure 5).

L'ajustement du modèle mathématique de croissance de Baranyi [5] à ces données expérimentales nous a permis de déterminer les temps de latence induits par ces conditions.

Pour le profil thermique correspondant au stockage des échantillons à -14°C pendant 4 heures puis à - 9°C pendant une semaine, le temps de latence donné par le modèle est de 44,8 h ± 8.5, et de 15,9 h ± 9.1 pour les échantillons stockés à -5°C pendant une semaine.

Ces résultats n'étant obtenus qu'à partir d'un seul essai, leur interprétation reste à confirmer. Il semblerait que la conservation des échantillons aux températures négatives de -14°C/-9°C ait un effet plus marqué sur le temps de latence de *Listeria monocytogenes* que le profil à –5°C.

Actuellement, ces expérimentations sont reproduites au laboratoire pour valider ces premiers résultats et nous permettre de conclure de façon plus globale sur l'incidence réelle des pratiques de préparation du saumon fumé sur la durée de la phase de latence de *Listeria monocytogenes*.

Les données publiées dans la littérature, relatives à l'effet de la température sur le temps de latence de *Listeria monocytogenes* [6], montrent qu'il existe un effet significatif de celle-ci sur la durée de la latence. Ainsi, à titre d'exemple, les temps de latence mesurés à 5°C et 10°C dans un milieu de culture liquide à 0.9 M de NaCl sont respectivement de 110 h et 80 h. Toutefois, ces résultats, obtenus dans des conditions de croissance très favorables (milieu de culture riche en nutriments) et à partir de micro-organismes cultivés à la température optimale de croissance de 37°C, sont assez éloignés des conditions naturelles de contamination et semblent surévaluer l'effet de la température sur la durée de la latence.

Conclusion

Les profils thermiques du process de préparation du saumon fumé rencontrés dans le domaine industriel varient principalement au niveau de l'étape de tranchage ; les différences observées sont directement liées au matériel de tranchage utilisé.

Les résultats de cette étude relatifs à l'incidence du froid sur la croissance de *Listeria monocytogenes* et plus spécifiquement sur son temps de latence, montrent que les différents profils thermiques à températures négatives propres à une technologie de tranchage n'ont en réalité que peu de conséquences sur la durée de la latence de ce micro-organisme dans l'aliment. ■

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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. [Devlieghere, F., Geeraerd, A.H., Versyck, K.J., Vandewaetere, B., van Impe, J., Debevere, J., 2001. Growth of *Listeria monocytogenes* in modified atmosphere packed cooked meat products: a predictive model. Food Microbiology 18, 53–66.] and modified by Giménez and Dalgaard [Giménez, B., Dalgaard, P., 2004. Modelling and predicting the simultaneous growth of *Listeria monocytogenes* and spoilage micro-organisms in cold-smoked salmon. Journal of Applied Microbiology 96, 96–109.] 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 (2003). Briefly, primary models describe the evolution of a population of microorganisms 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

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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 *L. 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/100 g) 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 physico-chemical 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/70 μm film (Euralpack, Saint Pierre du Perray, France), with low transmission rates: 30–40 $cm^3\ m^{-2}\ day^{-1}\ bar^{-1}$ 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±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 stomacher. *L. monocytogenes* was enumerated by plating onto Palcam agar (AES) appropriate dilutions of the disks in tryptone salt. Plates were incubated 48±2 h at 37±1 °C. Cell counts were calculated per square centimetre of salmon surface, so that population densities of *L. monocytogenes* were expressed as log cfu/cm^2. The theoretical initial contamination, calculated from the contamination level for the inoculation suspension, is 3.5 log cfu/cm^2, which is approximately equivalent to 4 log cfu/g.

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

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

2.4. Estimation of growth rates

Each *L. monocytogenes* growth curve was fitted by the model of Baranyi and Roberts (1994) and by the embedded model without lag-phase, using least-squares nonlinear regression. An *F*-test was performed to compare both models, with $\alpha = 0.05$. In all cases but one (Batch E, 8 °C), the lag time was not significant and the model without lag phase was then selected. This is easily explained by the history of the strain, as the preculture temperature was 10 °C. The parameters of the chosen model were also estimated using robust nonlinear regression, as detailed in Miconnet et al. (2005). All calculations were performed with the software Matlab 6.5 (MathWorks).

When it was appropriate, the same estimation was performed for the food flora, obtained through storage trials.

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

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

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

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

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

- 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):

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

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).

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

3. Results and discussion

3.1. Physicochemical characteristics

Forty French commercial products were surveyed and analysed for pH, salt contents, water contents, water-phase salt contents, and phenolic contents (see Table 1). Results were compared with those of Leroi et al. (2001), on 13 French commercial products, supposed to be representative of the French traditional production and Espe et al. (2004), on 48 French commercial products, produced by four commercial smoking-houses. Water and salt contents were very similar with those observed by Leroi et al. (2001). Significant differences were observed between both studies regarding two factors: pH and phenolic contents. Indeed, Leroi et al. (2001) measured "initial" pH in early shelf life, whereas pH-values were measured later in the shelf life in the present study, which may explain the slightly lower results. Last, Leroi et al. (2001) observed lower phenolic contents, as low as 0.27 mg/100 g, and none above 1.1 mg/100 g. This difference may be explained by an unexpected sampling or experimental bias or could reflect a recent evolution of the French market. Statistical comparisons could not be performed using results of Espe et al. (2004), as raw results had not been published, but results appeared close to the ones of the present survey.

The measured physicochemical characteristics of the 5 specific products used for challenge tests (see Table 1) were all within the ranges of those of the commercial products (at least from one of the three surveys, see Table 1). However, one batch (C) appeared relatively lightly salted. The 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. Fig. 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, etc.) 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 for publication).

Table 1
Physicochemical characteristics (pH, salt content, water-phase salt content calculated from salt and water contents, and phenolic content) of cold-smoked salmon

	pH	Salt content (g/100 g)	WPS (g/100 ml)	Water content	P=phenolic content (mg/100 g)
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., 2004)					
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

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.

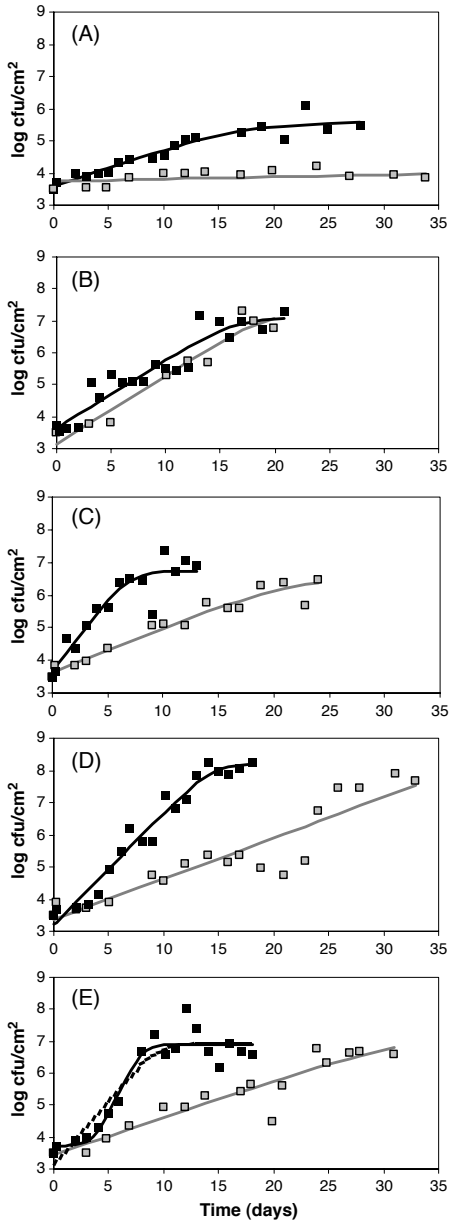

Fig. 1. Growth curves of *L. monocytogenes* on cold-smoked salmon (log cfu/cm^2), after surface-contamination of batches A (A) to E (E). Experimental results (grey squares: 4 °C, black squares: 8 °C) were fitted by least-squares regression with the selected primary model without lag (grey solid line: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

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/100 g, 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.

The similarity of growth rates at 4 °C and 8 °C of batch B was unexpected. This unexpected result might be (at least partly) due to estimation uncertainty. Note that the obtained growth curves, both at 4 °C and 8 °C, are far from ideal exponential growth curves. The uncertainty on the estimation of growth rates is then high. As discussed in Miconnet et al. (2005), surface growth curves are often less satisfactory than crushed growth curves, due to an heterogeneity between packs, which are not homogenised in the 1st case, whereas they are in the latter case.

This unexpected result of batch B could also be explained by the within-batch variability. Indeed, there could have been a difference between the sub-batch used for the growth curve at 4 °C and the sub-batch used for the growth curve at 8 °C.

Moreover, at 4 °C, the growth rate in batch B is higher than in batch E, and the opposite is observed at 8 °C, whereas batches B and E have similar physicochemical characteristics. This may be explained by the estimation uncertainty discussed above. It also illustrates the fact that the measured physico-chemical characteristics probably do not account for all the between-batches and within-batch variability.

Table 2
Estimated and predicted growth rates (day^{-1}) of *L. monocytogenes* on cold-smoked salmon at 4 °C and 8 °C

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

Estimations were obtained fitting each growth curve using the chosen model (see Fig. 1) and non-linear least-squares and robust regression (Miconnet et al., 2005). 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).

[a] The estimation of 1.63 day^{-1} for batch E at 8 °C is obtained with the 4-parameter Baranyi model. With a null lag time, the estimated growth rate is 0.92 day^{-1}.

3.3. Growth of the food flora in specific products

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

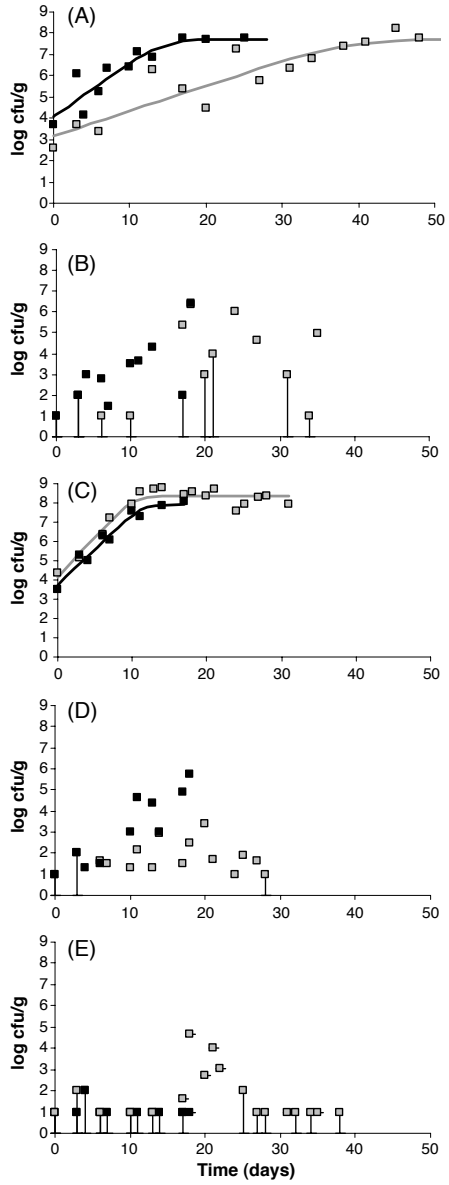

Fig. 2. Growth curves of the mesophilic food flora in cold-smoked salmon (log cfu/g), batches A (A) to E (E). Experimental results (grey squares: 4 °C, black squares: 8 °C) below a limit of quantification are represented at this limit with a vertical bar. For two batches, A and C, growth curves were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

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 and Bagi, 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 the growth rates in these batches were not influenced by the background flora. On the contrary, the observed maximal population densities observed by *L. monocytogenes* may be lower than those obtained in absence of this predominant background flora.

For the three industrial batches, denoted B, D, and E, the initial population was so low, that most packs could not be enumerated using standard techniques (with a quantification threshold at 10 cfu/g). It is then impossible for these batches to estimate the growth rates. However, in the case of batch B, it cannot be excluded that the growth at 4 °C could be close

to the growth at 8 °C (as enumeration results are similar at the 18th day). This could confirm that there was a difference between the physicochemical characteristics of the sub-batch used at 4 °C and the sub-batch used at 8 °C. This could also be explained by species with a low optimal temperature, as for the food flora of batch C discussed above. For these three batches, *L. monocytogenes* was strongly predominant in the challenge tests and we can then exclude that any competition effect occurred during the experiments. However, as realistic initial contamination levels of *L. monocytogenes* are very low (see Beaufort et al., submitted for publication), competition should be taken into account when predicting the evolution of *L. monocytogenes* in naturally contaminated products, even for these batches with a relatively initial level in background flora.

3.4. Comparison and validation of L. monocytogenes secondary models

Published secondary models were evaluated in this study. Predicted growth rates are compared with estimated growth rates of the present study in Table 2. Predictions of models 1 and 2 were much lower than the observations (i.e. fail-dangerous). Predictions of models 3 and 4 were more consistent with estimated growth rates, even if model 3 tended to be fail-dangerous at 4 °C. The estimated growth rate for batch E at 8 °C, 1.63 day^{-1}, appears relatively high when compared with 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} is the MIC-value for WPS, calculated from the minimal a_w of each model, using Eq. (1), P_{max} is the phenolic MIC-value, k equals 1 or 2, and μ_{ref} is 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/100 g = 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 nonsmoked 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.

Eq. (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/100 g.

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 nonsmoked salmon. Eleven secondary models were tested. Among the four models taking into account the phenolic effect, model 4 was the most accurate model (lowest A_f-value) on each data set. It was slightly biased, in a fail-safe way, ($B_f > 1$) 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, B_f-values close to 1.0 were expected and were indeed obtained. More surprisingly, the A_f-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

Table 3
Parameters of three models in a unified and simplified equation (see Eq. (2) in the text)

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/100 mL)	13.1	11.6	10.7
P_{max} (mg/100 g)	1.25	2.81	2.81
k	2	1	1

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 Fig. 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 non-smoked salmon (Peterson et al., 1993; Pelroy et al., 1994; Niedziela et al., 1998)

	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

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). Model 1′: Augustin and Carlier (2000a), without phenolic effect ($P_{max}=\infty$). Model 2′: Augustin and Carlier (2000b), without phenolic effect ($P_{max}=\infty$). Model 3′: Ross (FAO/WHO, 2004). Model 4′: Devlieghere et al. (2001). Model 5: Pathogen Modeling program (USDA, 2001). Model 6: Growth Predictor (IFR, 2004). Model 7: FDA (2003).
[a] For models 1 and 2, the criteria are not defined, as some predicted growth rates are equal to 0. To obtain numerical values, null predictions were replaced by 0.01 day^{-1}.

improved. Thus, the effect of organic acids was only taken into account through the initial pH, whereas the production of lactic acid by the background flora could have been specifically considered. The measurement of WPS is probably not sufficient to study the water activity, as sucrose, measured by Espe et al. (2004) in French products, or other solutes may also lower it. The within-batch variability, e.g. the variability of the WPS due to this more or less equal repartition of the salt (which could depend on the salting method), was not considered in this study but could have a great impact, as suggested by some unexpected results. Last, Brocklehurst (2003) reviews numerous studies which demonstrate that the microstructure of the food impacts the microbial growth.

In a broader context, additional preservatives, which are forbidden in France and were not used in the batches selected for these experiments, may be taken into account. For example, when they have been used, nitrites had a significant effect (Pelroy et al., 1994).

Then, additional sources of between-product and within-product variability have still to be investigated, before a full validation of secondary models based on physicochemical characteristics. Investigation of secondary models based on an alternative description of the variability could also be valuable.

3.5. Phenolic effect

The highly fail-dangerous characteristic of models 1 and 2 can be easily explained, as Augustin and Carlier (2000a,b) based their estimations of the phenolic MIC-value on experimental results in which a phenolic concentration of 1.25 mg/100 ml in broth was inhibitory for *L. monocytogenes* (Membré et al., 1997), whereas a concentration of 2 mg/100 g is not, at least at 8 °C, in our results. This apparent contradiction may be explained by a difference between the behaviour of phenolic compounds in broth versus fish. The solubility of these compounds in the 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 which is not explained by the measured physicochemical factors.

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Modelling the effect of a temperature shift on the lag phase duration of *Listeria monocytogenes*

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Abstract

The aim of this work is to study and model the effect of a temperature shift on h_0, the product of the growth rate by the lag phase duration ($\mu\lambda$). Our work is based on the data of Whiting and Bagi [Int. J. Food Microbiol. 73 (2002) 291], who studied the influence of both the pre-incubation temperature (T_{prior}) and the growth temperature (T_{growth}) on λ values of *Listeria monocytogenes*. We introduce a new model to describe the evolution of the parameter h_0 as a function of T_{prior} and T_{growth}, and compare it to Whiting and Bagi's published polynomial model that describes the influence of T_{prior} and T_{growth} on λ independently of μ. For exponential as well as stationary phase cells, h_0 increases almost linearly with the magnitude of the temperature shift. A simple linear model of h_0 turns out to be more suitable to predict λ values than a polynomial model of λ.

Keywords: Lag phase duration; Work to be done; Temperature shift; *Listeria monocytogenes*

1. Introduction

A lag phase corresponds to a transition period during which microbial cells adjust to their new environment before exponentially growing. Such an adjustment period is generally observed at the beginning of experimental microbial growth kinetics, when the culture medium is inoculated by a preculture. In food microbiology, a lag phase might occur just after a contamination from the environment to the product, or when a contaminated food product undergoes important environmental fluctuations. A better understanding of the factors affecting the lag phase duration might provide ways of delaying or preventing growth of undesirable microbial populations.

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Unfortunately, the lag phase duration is much more difficult to predict than the specific growth rate, as it does not only depend on current conditions, but also on previous environmental conditions of the bacterial cells and on their physiological state. Many authors have reported a strong influence of the pre-incubation temperature on the lag phase duration (Walker et al., 1990; Buchanan and Klawitter, 1991; Hudson, 1993; Bréand et al., 1997, Dufrenne et al., 1997; Membré et al., 1999; Augustin et al., 2000; Whiting and Bagi, 2002). As an example, bacterial cells previously cultured at low temperatures have a reduced lag at low temperatures compared with cells previously cultured at high temperatures (Walker et al., 1990; Membré et al., 1999; Whiting and Bagi, 2002).

Some authors model the lag phase duration (λ) independently of the specific growth rate (μ) (or generation time) (for a review see Delignette-Muller, 1998). They generally propose polynomial λ models developed from growth kinetics of cells previously cultured at a favourable high temperature. Consequently, when these models are used to predict the growth of an environment contaminant in a refrigerated food product, λ is overestimated. Membré et al. (1999) then suggest that in such studies, microorganisms should be previously cultured at low temperatures, in order to mimic the processes of contamination in industry.

Other authors assume that the product $h_0=\mu\lambda$ does not depend on the growth conditions, but only of the pre-incubation conditions (Baranyi and Roberts, 1994; McKellar, 1997; Augustin and Carlier, 2000). Under this assumption, λ may be simply predicted from the predicted value of μ and from the constant h_0 for given pre-incubation conditions (Rosso, 1995). This product h_0 was described as the "work to be done" by the cells during the lag phase to prepare for the exponential growth (Robinson et al., 1998; Pin et al., 2002). If no effect of the growth temperature on h_0 is generally reported in the published studies, an effect of the medium pH on h_0 was observed in several studies (Delignette-Muller, 1998) as well as an effect of CO_2 and O_2 concentrations (Pin et al., 2002).

Our work is based on data from Whiting and Bagi (2002), who studied the influence of the pre-incubation temperature (T_{prior}) and the growth temperature (T_{growth}) on λ values of *Listeria monocytogenes*. We propose a new model to describe the evolution of the parameter h_0 as a function of T_{prior} and T_{growth}, and compare it to Whiting and Bagi's (2002) published polynomial model that describes the influence of T_{prior} and T_{growth} on λ independently of μ. Our work was previously presented during the fourth International Conference "Predictive Modelling in Foods" (Delignette-Muller et al., 2003).

2. Materials and methods

2.1. Experimental data

In this study, we used the experimental data reported by Whiting and Bagi (2002) which concerned the growth of *L. monocytogenes* Scott A. Cells were precultured in a brain heart infusion (BHI) broth at different temperatures (4, 8, 15, 28 and 37 °C) to the exponential growth or the stationary phases. These cells were then immediately transferred to BHI broth at various temperatures (4, 8, 15, 28 and 37 °C), to reach an initial level of approximately 10^3 cfu/ml. The growth curves were obtained by viable count enumeration and fitted by the three-phase linear model (Buchanan et al., 1997) in order to estimate λ and μ. Growth rate values were reported only for exponential phase cells and no effect, neither of the initial physiological state nor of the T_{prior} temperature, was reported on μ. In our study, the values of h_0 were then calculated from the reported values of λ and from the values of μ estimated by the square root model proposed by Whiting and Bagi (2002).

2.2. Fitting procedures and statistical methods

All statistical calculations were computed using the R Software version 1.6.1 (Ihaka and Gentleman, 1996). Fits of models were performed by nonlinear regression by using the least-squares criterion (Bates and Watts, 1988). Nonlinear regression was computed with the *nls* package available with R. The performance of the models was evaluated by using a comparison of root mean square error (RMSE):

$$\mathrm{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}$$

where n is the number of data points, y_i is the ith observed value and $\hat{y}_i$ is the ith predicted value.

Comparisons of nested models were performed using an F test (Bates and Watts, 1988).

$$F_{obs} = \frac{(n - p_f)(RSS_p - RSS_f)}{(p_f - p_p)RSS_f}$$

where n is the number of data points, p_f is the number of parameters of the full model, p_p is the number of parameters of the partial model, RSS_f is the residual sum of squares of the full model fit, and RSS_p is the residual sum of squares of the partial model fit. The observed F value must be compared with a theoretical F value with $v_1 = p_f - p_p$ and $v_2 = n - p_f$ degrees of freedom.

3. Results

3.1. Modelling of h_0

The values of h_0 for various values of T_{prior} and T_{growth} and for both initial physiological states are reported in Figs. 1 and 2. The h_0 mean value appears to be clearly less for exponential phase cells compared with stationary phase cells. For each value of T_{prior}, values of T_{growth} have an effect on h_0, especially for extreme values of T_{prior}. As we can see in Fig. 1, for low T_{prior} temperatures, h_0 increases with T_{growth} and for high T_{prior} temperatures, h_0 decreases with T_{growth}. A first result is then that the assumption that h_0 is a constant for a given pre-incubation temperature is not supported by these data.

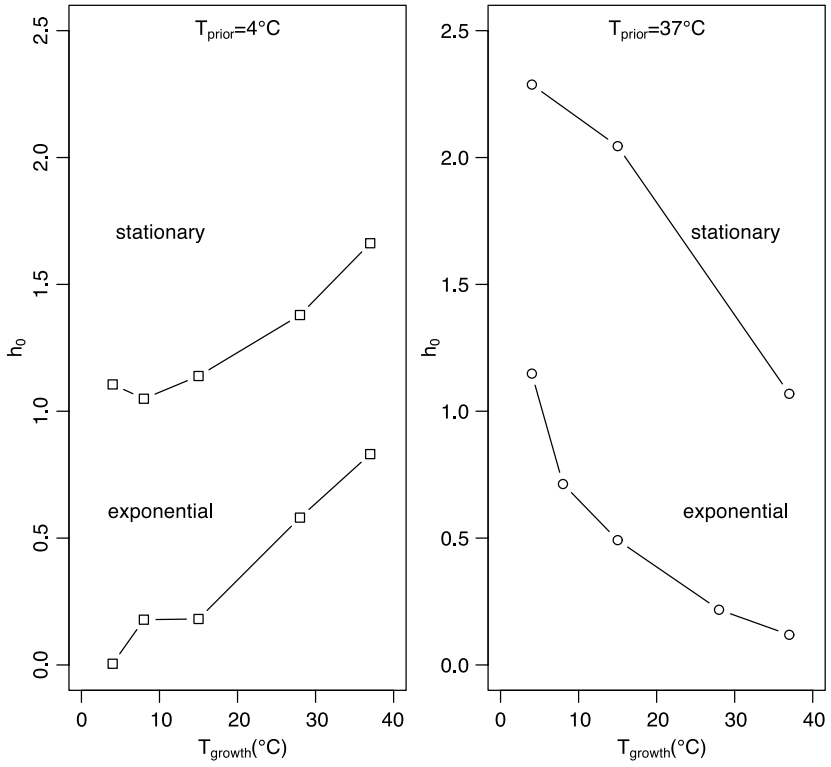

Fig. 1. The values of h_0 for exponential and stationary initial physiological states, as a function of the T_{growth} temperature for two extreme T_{prior} temperatures.

In Fig. 2, all the values of h_0 are plotted against the temperature difference ($T_{growth}-T_{prior}$). Globally, h_0 seems to increase with the magnitude of the temperature shift. This increase seems identical for cells in exponential or stationary state, but the minimum value of h_0, corresponding to no temperature shift, is roughly null for exponential phase and just above one for stationary phase cells. We propose a simple model (Model 1) to describe the global trend observed in Fig. 2. This model describes four linear segments and is characterized by three parameters:

$$h_0 = \begin{cases} b_1\left(T_{growth} - T_{prior}\right) & \text{for } T_{growth} > T_{prior} \text{ and exponential state} \\ a + b_1\left(T_{growth} - T_{prior}\right) & \text{for } T_{growth} > T_{prior} \text{ and stationary state} \\ -b_2\left(T_{growth} - T_{prior}\right) & \text{for } T_{growth} < T_{prior} \text{ and exponential state} \\ a - b_2\left(T_{growth} - T_{prior}\right) & \text{for } T_{growth} < T_{prior} \text{ and stationary state} \end{cases} \quad (1)$$

where a is the theoretical minimal value of h_0 for stationary phase cells, b_1 ($^{\circ}C^{-1}$) is the slope of the linear increase of h_0 for a positive temperature shift and b_2 ($^{\circ}C^{-1}$) is the slope of the linear decrease of h_0 for a negative temperature shift. This model was globally fitted to the data by nonlinear regression. An equivalent fit could be obtained by linear regression after introducing some dummy variables, since the model is actually linear in the parameters (Draper and Smith, 1998). The fitted model is represented in Fig. 2. The fitted values of a, b_1 and b_2 and their 95% marginal confidence intervals are 1.06 ([0.97–1.16]), 0.022 ([0.018–0.026]) and 0.031 ([0.027–0.036]).

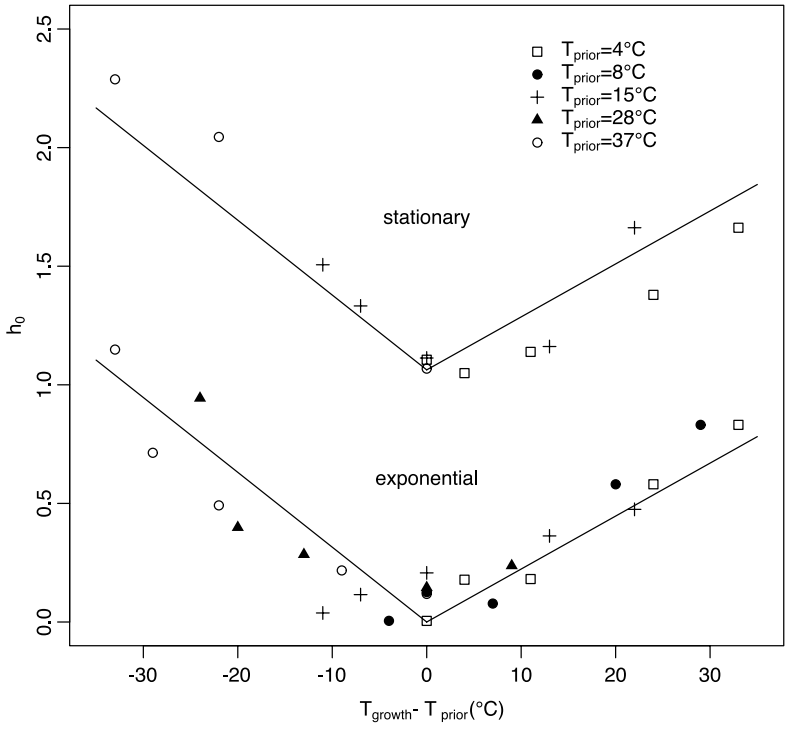

Fig. 2. The values of h_0 for exponential and stationary initial physiological states as a function of the temperature shift ($T_{growth}-T_{prior}$) for all the T_{prior} temperatures tested, with Model 1 fitted on these values (—).

3.2. Models comparisons

Model 1 was compared with two other models for the prediction of the λ values of the data set. Model 2 is defined as the association of the two polynomial models proposed by Whiting and Bagi (2002) for exponential and stationary initial physiological states, each with six parameters.

$$\lambda = \begin{cases} 10^{-0.8988+0.04237T_{\text{prior}}+0.02903T_{\text{growth}}+0.000657T_{\text{prior}}^2+0.000128T_{\text{growth}}^2-0.00289T_{\text{prior}}T_{\text{growth}}} & \text{for exponential state} \\ 10^{1.619648-0.000337T_{\text{prior}}-0.081914T_{\text{growth}}+0.000350T_{\text{prior}}^2+0.001251T_{\text{growth}}^2-0.000591T_{\text{prior}}T_{\text{growth}}} & \text{for stationary state} \end{cases} \quad (2)$$

In Model 3, h_0 is classically assumed independent of T_{growth}. As no obvious effect of T_{prior} on the h_0 mean value was previously observed, h_0 is assumed constant for each physiological state and fixed to the mean observed values for each state. So Model 3 includes two parameters.

$$h_0 = \begin{cases} 0.37 \text{ for exponential state} \\ 1.42 \text{ for stationary state} \end{cases} \quad (3)$$

For Models 1 and 3, the values of λ were calculated from the predicted values of h_0 and the values of μ predicted by the square root model given by Whiting and Bagi (2002):

$$\mu = \ln(10) \times \left(0.07427 + 0.0174T_{\text{growth}}\right)^2 \quad (4)$$

The theoretical values of h_0 predicted by Model 3 and by the association of Model 2 and Eq. (4) are respectively reported in Figs. 3 and 4, against the observed values of h_0 for exponential and stationary phase cells. We can see

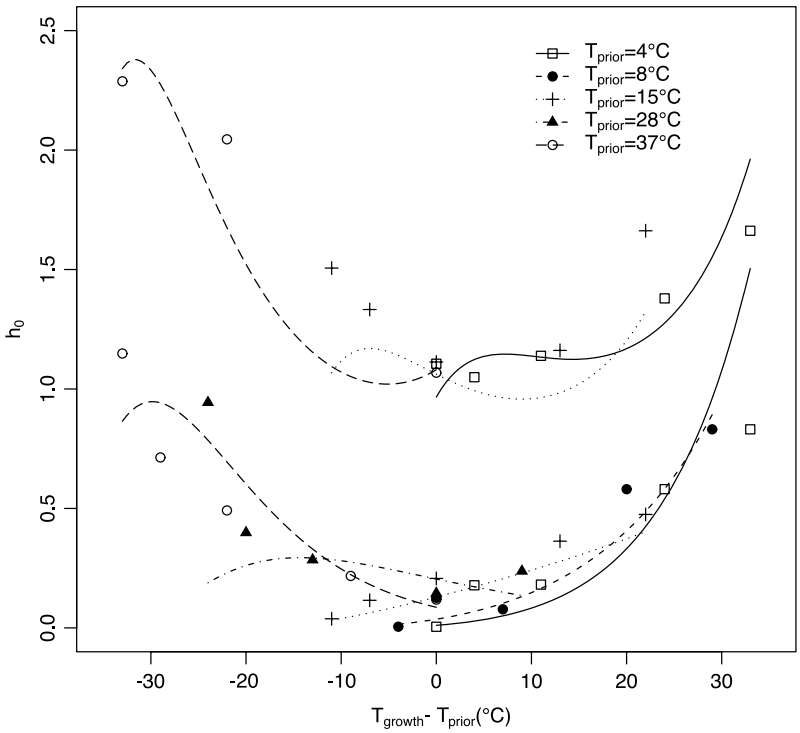

Fig. 3. The theoretical values of h_0 calculated as the product of the values of λ predicted by Model 2 by the values of μ predicted by the square root model (Whiting and Bagi, 2002) superimposed with all the values of h_0 observed for exponential and stationary initial physiological states as a function of the temperature shift ($T_{\text{growth}}-T_{\text{prior}}$).

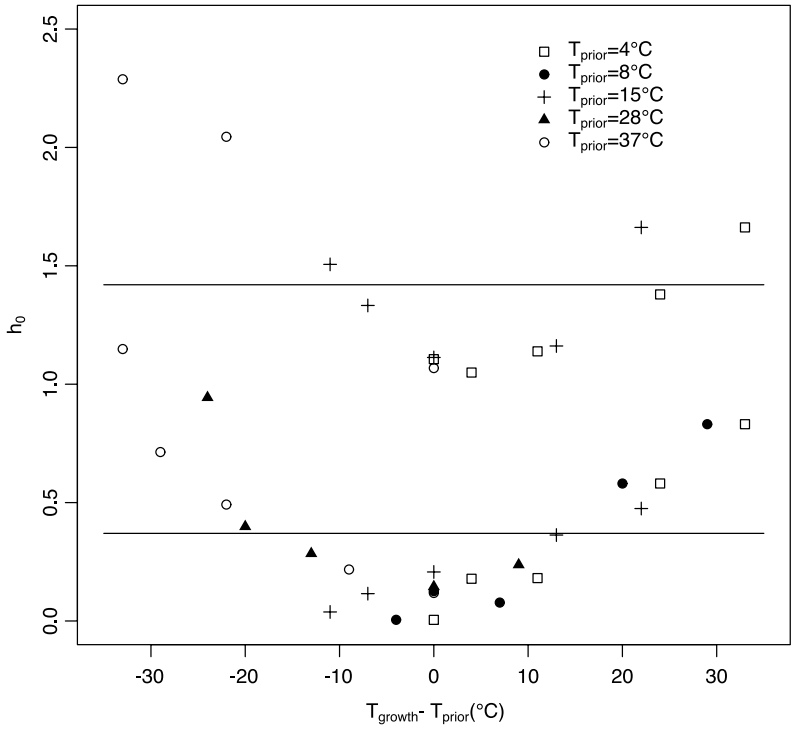

Fig. 4. The theoretical values of h_0 predicted by Model 3 (—) superimposed with all the values of h_0 observed for exponential and stationary initial physiological states as a function of the temperature shift ($T_{growth}-T_{prior}$).

comparing Figs. 2 and 4 that Model 1 gives a much better description of the evolution of h_0 than Model 3, with one more parameter only. Now comparing Figs. 2 and 3, Model 1 seems to give a more robust description of the evolution of h_0 than Model 2. Especially, the maximal theoretical values of h_0 described by Model 2 for several T_{prior} temperatures near the minimal difference $T_{growth}-T_{prior}$ does not seem biologically sounded.

In order to compare the three models for the prediction of the lag phase durations, the Root Mean Square Error (RMSE) on the predicted values of λ were computed. RMSE values obtained with Models 1, 2 and 3 are respectively 1.66, 3.23 and 5.14. In terms of RMSE, Model 1 with three parameters gives better predictions than Model 2 with 12 parameters, and much better predictions than Model 3 with two parameters. So even if Model 1 was not directly fitted on λ values, it gives good predictions of λ values in comparison with Model 2.

4. Discussion

The study of the dataset of Whiting and Bagi (2002) shows interesting results concerning the evolution of h_0 with the magnitude of the temperature shift. For both exponential and stationary initial physiological states, h_0 increases almost linearly with the magnitude of the temperature shift. A roughly similar trend is observed for both physiological states, but with different minimal h_0 values. When there is no temperature shift, the values of h_0 are around 1 for stationary phase cells and roughly nil for exponential phase cells. A simple linear model describing this approximated evolution of h_0 with only three parameters turns out to give better predictions of the values of λ than the polynomial model developed by Whiting

and Bagi (2002) with 12 parameters. Modelling h_0 instead of μ and λ separately appears here simpler and more efficient.

In Whiting and Bagi's work (2002), three other initial physiological states of the cells were studied. After the first growth cultures at various T_{prior} temperatures, additional cells were suspended in a dilute broth, dessicated or frozen. Second cultures of these cells were then done at various T_{growth} temperatures. For starved and frozen cells, an increase of h_0 with the magnitude of the temperature shift was also observed, but mainly for a positive temperature shift, and with far more variability around the trend than with exponential and stationary phase cells. Regarding this variability, it does not seem reasonable to fit any model on the observed values of h_0. For dried cells, an increase of h_0 with the magnitude of the temperature shift was observed only for a negative temperature shift, and with a few points only. So it does not seem possible to generalize the results observed on exponential and stationary phase cells to cells grown to the other physiological states studied by Whiting and Bagi (2002).

Coming to the observed evolution of h_0 for exponential and stationary phase cells, if h_0 is regarded as the "work to be done" by the cells during the lag phase to prepare for the exponential growth, it may then be interpreted as the sum of the work to adapt to a new environment and the work to emerge from the stationary state. In the studied data, the only change between pre-growth and growth conditions concerns temperature. If another growth factor is changed, such as the growth medium, another term corresponding to the work to adapt to this new medium may be added to h_0. Moreover, the definition of the stationary physiological state is not unique. Augustin et al. (2000) reported a continuous increase of h_0 as a function of the time spent in the stationary phase for a given couple (T_{prior}, T_{growth}). The gap between h_0 values for exponential and stationary initial physiological states may then be variable. Moreover, in Whiting and Bagi's study (2002), the temperature transition between T_{prior} and T_{growth} was instantaneous. A slower temperature transition might have a lower impact on h_0 (Whiting and Bagi, 2002). With all these comments in mind, we should be very cautious before using any model to predict λ values from T_{prior} and T_{growth} temperatures only. Such a model might take into account various other experimental conditions.

The existence of a temperature shift cut-off above which h_0 is affected was reported in the literature (Buchanan and Klawitter, 1991; Augustin et al., 2000). The existence of such a cut-off is not obvious in this study, but neither could it be excluded. The existence of a temperature shift cut-off appears more likely on data related to the exponential state (Fig. 2). Ng et al. (1962) and Shaw (1967) observed that temperature shifts within the normal physiological temperature range (NPTR, approximately 20–37 °C for *Escherichia coli*) have little effect on the lag phase duration. It seems difficult to check in this study as most of the data points correspond to shifts from or to a temperature outside this NPTR. Shaw (1967) also studied on mesophilic and psychrophilic yeasts the effect of negative temperature shifts from different moderate T_{prior} temperatures to one low T_{growth} temperature below the NPTR. He observed a linear relationship between the magnitude of the shift and the lag phase duration. This is concordant with our results, since h_0 may be assumed proportional to λ for a constant T_{growth} temperature.

Our results are globally concordant with the observations made by Hudson (1993) on *Aeromonas hydrophila* JAH4 cells initially in exponential state. In their experiment, four T_{prior} temperatures (from 5 to 35 °C) were crossed with four T_{growth} temperatures (from 5 to 35 °C). By calculating the values of h_0 from their reported results, we can see a global increase of h_0 with the magnitude of the temperature shift. On the other hand, our results are discordant with the observations made by Mellefont and Ross (2003) on *E. coli* SB1 cells initially in late exponential state. In their experiment, two extreme T_{prior} temperatures (10 and 44 °C) were crossed with 23 T_{growth} temperatures. An increase of h_0 was observed only for a negative temperature shift in their study, but no increase was observed for a positive shift from 10 °C.

More generally, a linear increase of h_0 with the magnitude of the temperature shift is not usually observed and h_0 is sometimes considered independent of it. This might be due to a stronger impact of additional variables such as pH and salt concentration in some studies (Delignette-Muller, 1998) and by a smaller range of temperature shifts (Pin et al., 2002). Moreover, when pre-incubation occurs at sub-optimal

conditions, as it is the case for most of the publications (Delignette-Muller, 1998), h_0 appears to be approximately constant for different post-incubation temperatures, but for inimical pre-incubation conditions, h_0 is no longer independent of temperature. A potential effect of the temperature shift on the lag phase duration should then be taken into account. It would be very interesting to perform experiments similar to that of Whiting and Bagi (2002) on other microbial species, in order to see if the global linear increase of h_0 with the magnitude of the temperature shift can be confirmed. From such data, the modelling of the parameter h_0 should be performed, as it may lead to simpler, better and more understandable models than the independent modelling of the lag phase duration.

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Reflections on the use of robust and least-squares non-linear regression to model challenge tests conducted in/on food products

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Abstract

In this research, we question the straight-forward use of the classical sum of squared error criterion for identifying the typical parameters of a primary model (like growth rate μ_{max} and lag time λ) when applied to growth curves obtained in and on food products. Firstly, we base our reflections on 62 *Listeria monocytogenes* laboratory challenge tests collected in various environments (broth, crushed cold-smoked salmon, and surface of cold-smoked salmon slices). Whereas growth data in broth resulted in residual values consistent with a Gaussian distribution, growth data in the crushed product and even more on the surface of slices appeared different. Secondly, we propose the use of an alternative so-called robust non-linear regression method suitable when experimental error is non-normally distributed, which seems, according to this research, typical for microbial challenge tests in/on food products, and which lead to apparent outliers or leverage points in the experimental data. Properties of the robust regression procedure are illustrated on simulated data first, whereafter its use on the considered challenge tests is illustrated. To conclude, reflections on the assumptions and related realism underlying challenge tests and recommendations for fitting growth curves obtained in and on food products are presented.

Keywords: Predictive microbiology; Primary model; Challenge tests; Structured food products; *Listeria monocytogenes*

1. Introduction

A typical microbial growth curve is obtained by a batch culture in an homogeneous, liquid environment, contaminated by an initial number of N_0 microbial cells. Schematically, it has three distinct phases: (i) an initial lag phase, the duration of which is denoted λ, and during which the N_0 microbial cells adapt to their new environment, (ii) an exponential growth phase, where cells are multiplying at a certain rate μ_{max}, (iii) and a stationary phase, where the maximum population density N_{max} is reached and growth ceases. Two additive transition phases can also be considered be-

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tween them and a (rapidly or slowly occurring) inactivation phase follows this growth curve. Various equations, the so-called primary models, have been proposed to describe such growth curves as a function of time t and thus to estimate N_0, λ, μ_{max} and N_{max} (or equivalent parameters).

As stated by McKellar and Lu (2003a), the concept of the primary model is fundamental to the field of predictive microbiology, which aims to predict the behaviour of spoilage or pathogenic microbial flora in food products (see McMeekin et al., 1993; Ross, 1999; McKellar and Lu, 2003b). Primary models are combined with secondary models (Whiting and Buchanan, 1993), describing the influence of the environment (mostly temperature, possibly pH, water activity, content in growth inhibitors) on the growth rate μ_{max} (and possibly on the lag time λ and on the maximum population density N_{max}).

Challenge tests, studying growth kinetics of deliberately added micro-organisms, are an important source of information in food microbiology. They are among the basic tools for determining the ability of a food to support the growth of spoilage organisms or pathogens, and to determine the potential shelf-life of perishable food products. More recently, they have been used in predictive microbiology (Brocklehurst, 2003; Wilson et al., 2002).

The estimated primary model parameters, especially the growth rates, can then be used either to create new secondary models (e.g. Rosso et al., 1996; Augustin and Carlier, 2000a,b; Oscar, 2002; FDA, 2003; Pinon et al., 2004; Giménez and Dalgaard, 2004) or to validate predictions of independent secondary models (e.g. Neumeyer et al., 1997; te Giffel and Zwietering, 1999; Devlieghere et al., 2000; Castillejo-Rodriguez et al., 2002; Pinon et al., 2004; Giménez and Dalgaard, 2004).

The classical way of identifying the parameters of a primary model (or any other regression model) is by minimizing the sum of squared errors between the data points and the model prediction. The parameter set corresponding to this classical least-squares error criterion is equivalent to a so-called maximum likelihood estimator (i.e., the parameter set maximising the likelihood of the experimental data being generated by the parameterised model) if the residual errors can be assumed to be normally distributed. In the case of growth curves in a liquid microbiological medium (or broth), this non-linear regression has been quite extensively studied from a statistical point of view (e.g. Baty et al., 2002; Poschet et al., 2003; Baty and Delignette-Muller, 2004). However, in the specific case of challenge-tests performed on or in (possibly) non-homogenous and solid food products, the statistical hypotheses underlying this regression and the associated uncertainty have, as far as we know, never been investigated.

The objective of this research is to investigate the use of a classical primary model to describe different types of growth curves, with a special interest in the statistical properties of non-linear regression. Section 2 deals with the classical least-squares regression, which is tested on 62 laboratory growth curves. In Section 3, the robust regression method, which appears more adapted to specific cases of challenge tests, is proposed, validated on simulated realistic growth curves, and finally tested on some experimental growth curves. In the last section, discussion and conclusions regarding challenge tests and appropriate modelling procedures are formulated.

2. Classical least-squares regression

2.1. Materials and methods

2.1.1. Microbial data

Three different types of growth experiments of *Listeria monocytogenes* are used in this research: 9 curves in broth, 29 curves in crushed cold-smoked salmon, and 24 curves on the surface of cold-smoked salmon slices, i.e., a total of 62 curves.

For each experiment in broth, a *L. monocytogenes* strain was subcultured twice at 10 °C or 37 °C in TSB or TSB–Ye. An Erlenmeyer flask, containing 1 L of TSB or TSB–Ye, was inoculated by 1 mL containing the desired cellular concentration, and incubated in a thermostated water bath, at 4, 8, 12, or 37 °C, the medium being aerated and mixed, using a magnetic stirrer. At each of the n sampling times, an aliquot was retrieved from the culture using spinal needles in the cap of the flask. The number of viable cells in this aliquot was determined by plate-counting on TSA and population densities were expressed as log [cfu/mL].

For each experiment in crushed cold-smoked salmon, a *L. monocytogenes* strain was subcultured twice

at 10 °C in TSB. Cold-smoked salmon slices of a same production batch were crushed together, mixed, and then n small 10-g packs were separated. A 0.1-ml volume containing the desired cellular concentration was added to each pack, which was mixed thoroughly, vacuum-packaged in oxygen-impermeable film, and incubated at 4 °C or 8 °C. For some curves, there were $n/3$ sampling times and at each of them, three 10-g packs were analysed, whereas, for other growth curves, there were n sampling times and at each of them, one 10-g pack was analysed. Packs were pummelled with buffer in a stomacher homogeniser. The population density of *L. monocytogenes* was determined by plate-counting on Palcam, and expressed as log [cfu/g].

For each experiment on the surface of cold-smoked salmon slices, a *L. monocytogenes* strain was subcultured twice at 10 °C in TSB. An 89-mm disk was cut from each of n cold-smoked salmon slices of a same production batch. A 0.1-ml volume containing the desired cellular concentration was spread onto each disk, which was then folded, so that the inoculum was sandwiched between the two layers. The n vacuum-packaged disks were incubated at 4 °C or 8 °C. At each of the n sampling times, one disk was pummelled with buffer in a stomacher homogeniser. The population density of *L. monocytogenes* was determined by plate-counting on Palcam, and expressed as log [cfu/cm^2].

The common features of these three types of experiments are that (i) they were all performed in the same laboratory, between 1998 and 2003, using similar plate-counting procedures, (ii) they all consisted in the inoculation of one (or n) unit(s) with a strain of *L. monocytogenes* and then the incubation of this unit (or these n units) in isothermal conditions, (iii) the number of data points per growth curve, n, was relatively high (usually around 20).

The main differences between the three types are (i) the nature of the inoculated environment (liquid broth, or solid food, i.e., cold-smoked salmon, crushed or not), (ii) the number of different inoculated units per curve, as there was only one common flask in a broth experiment, whereas there were n different packs or n different disks in a challenge test, (iii) the homogenisation of the growth environment(s) before and/or along the experiment, as there was a constant homogenisation during a whole broth experiment, whereas the homogenisation was only partial in a challenge test in crushed food (as the food was mixed before separation into n samples, and each sample was separately mixed after inoculation), and there was none in a challenge test on the surfaces.

2.1.2. Primary models

As mentioned above, several primary models have been proposed in the literature, and a very recent overview of existing primary models can be found in McKellar and Lu (2003a). However, we focused on one single primary model, introduced by Baranyi and Roberts (1994), which is widely used (e.g. George et al., 1997; McClure et al., 1997; van Gerwen and Zwietering, 1998; Rodriguez et al., 2000; Coleman et al., 2003; Cornu et al., 2003; Panagou et al., 2003).

In its original, dynamic formulation, the model of Baranyi and Roberts (1994) reads as follows.

$$\frac{dN}{dt} = \frac{Q}{1+Q}\mu_{max}\left(1-\frac{N}{N_{max}}\right)N \quad (1)$$

with $N(t=0)=N_0$

$$\frac{dQ}{dt} = \mu_{max}Q \quad (2)$$

with $Q(t=0)=Q_0$.

The first differential equation describes the evolution of the microbial load N, for example in [cfu/mL]. The first factor on the right hand side of this equation is related to the adjustment of the microbial cells during the lag phase by means of a variable representing the physiological state of the cells Q [adimensional]. This variable Q is assumed to be proportional to the concentration of a (hypothetical) critical substance which is the bottle-neck in the growth process. The second factor expresses the exponential phase, with μ_{max} the growth rate [day^{-1}]. The third factor describes the transition to the stationary phase where the maximum microbial load N_{max} is attained. The second differential equation describes the evolution of Q, which increases exponentially.

For static conditions, and for a given set of initial conditions $N(0)$ and $Q(0)$, the following explicit algebraic version can be derived.

$$y = \frac{y_1 + y_2 + y_3}{\ln 10} \quad (3)$$

with

$$y_1 = \mu_{max} t + y_0 \ln 10 \quad (4)$$

$$y_2 = \ln\left[e^{-\mu_{max}(\lambda+t)}\left(-1 + e^{\mu_{max}\lambda} + e^{t\mu_{max}}\right)\right] \quad (5)$$

$$y_3 = \ln\left[e^{-\lambda\mu_{max} - y_{max}\ln 10}\left(-e^{y_0 \ln 10} + e^{\mu_{max} t + y_0 \ln 10} + e^{\lambda\mu_{max} + y_{max}\ln 10}\right)\right] \quad (6)$$

In these equations, y_0 indicates the initial base 10 log count [log cfu/ml], with $y_0 = (\ln N(0))/(\ln 10) = \log N(0)$, y_{max} the maximal base 10 log count [log cfu/ml], with $y_{max} = \log N_{max}$, μ_{max}, the growth rate [day^{-1}], and λ the lag phase [days], with $\lambda = (\ln(1 + [1]/[\ln Q(0)]))/(\mu_{max})$.

2.1.3. Estimation procedure

Fitting a growth curve leads to the estimation of the four parameters of the primary model. The parameter of main interest for us here was the growth rate, μ_{max}. The regression problem can be summarized by the following notations $y_i = f(x,t_i) + r_i$ for $i = 1,\ldots,n$, with y_i the ith observation for the dependent variable [log cfu/ml], f the regression model (i.e., the Baranyi and Roberts primary model), x the set of the four parameters (μ_{max}, y_0, λ, y_{max}), t the independent variable (the time), r_i the ith residual, and n the number of observations. Non-linear regression is the result of this minimization problem:

$$\min_x \sum_{i=1}^{n} \rho(r_i(x)) \quad (7)$$

where $\rho(u) = u^2$ for the least-squares regression.

The validity of this error criterion is based on four assumptions: (i) the regression equation (in this case, the Baranyi and Roberts growth model) used is correct, (ii) the independent variables (in this case, time expressed in days) are perfectly measured, (iii) the dependent variables (in this case, $y = \log N$) have an experimental error which is constant over the range studied, (iv) the dependent variables (in this case, $y = \log N$) are independent and identically distributed. The least-squares estimator is then *efficient* (i.e., the obtained parameter variance is the smallest possible) in the case of Gaussian errors, and is in these circumstances equivalent to a maximum likelihood estimator.

The primary Baranyi and Roberts model was adjusted to each growth curve separately, by minimizing the sum of squared errors, using the *lsqnonlin* function of the Matlab-Optimization Toolbox (The Mathworks Inc., Natick, United States). The parameter λ was constrained to be positive. To circumvent the possible problem of local minima, 120 different sets of initial estimates of the four parameters were used. On the basis of previous trials (data not shown), the 120 sets of initial parameters were the 120 combinations of one fixed value for y_0 (e.g. the initial log-transformed observation), one fixed value for y_{max} (e.g. the last log-transformed observation), one value between 0.2 and 2 with a step of 0.2 for μ_{max}, and one value between 0.5 and 6 with a step of 0.5 for λ. The trials demonstrated that this precaution was highly sufficient, and even in most cases unnecessary. At most, four local minima were identified and the global minimum was retained in the further analysis.

2.1.4. Evaluation of fit and distribution of residuals

For each of the 62 growth curves (9 curves in broth, 29 curves in crushed cold-smoked salmon, and 24 curves on the surface of slices), the quality of the fit was evaluated visually and through various criteria describing the residuals: (i) the estimated root mean square error (RMSE), (ii) the highest residual (in absolute value), (iii) the skewness-values, and (iv) the Kurtosis-values of the distribution of n residuals.

Six parametric distributions (Gaussian, 2-parameter logistic, Laplace, shifted Weibull, shifted Gamma, and shifted Lognormal) were fitted to each distribution of n residuals. Five of these functions are defined in Vose (2000), whereas the Laplace distribution is defined through its probability density function: $f(x) = 0.5e^{-k|x|}$ where $k = \frac{\sqrt{2}}{\sigma}$.

Kolmogorov–Smirnoff and Anderson–Darling goodness-of-fit tests (Vose, 2000) were performed. Additionally, simulations were performed to enable another global goodness-of-fit evaluation. We simulated 10 000 sets of 20 values sampled from a Gaussian distribution, the Kurtosis-values of these simulated data were calculated and compared with the Kurtosis-values of the empirical distributions, each of n residuals. The same simulations and comparisons were repeated with 10 000 sets of 20 values sampled from a two-parameter logistic parametric distribution and

with 10 000 sets of 20 values sampled from a Laplace parametric distribution.

All calculations were performed using Matlab.

2.2. Results

2.2.1. Pattern of growth curves

Fig. 1 presents four examples of growth curves. The growth curve in broth (Fig. 1a) exhibits the classical traits of a typical growth curve in broth, with the lag phase, exponential phase and stationary phase. The first growth curve in crushed food (Fig. 1b) is similar, even if the exponential growth phase appears slightly noisy. In the second growth curve in crushed food (Fig. 1c), the exponential growth phase is even more noisy, with slow-downs after the 10th and the 15th day, and a (visual) outlier point at the 19th day. Moreover, the experiment was (non-purposely) interrupted too early, and the stationary phase is poorly described. Last, outliers are also observed in the growth curve obtained on the surface of cold-smoked salmon slices (Fig. 1d). These four examples are representative for the overall set of data.

2.2.2. Quality of fit

Table 1 presents the quartiles of estimated root mean squares errors for each growth environment. Important differences related to the growth environ-

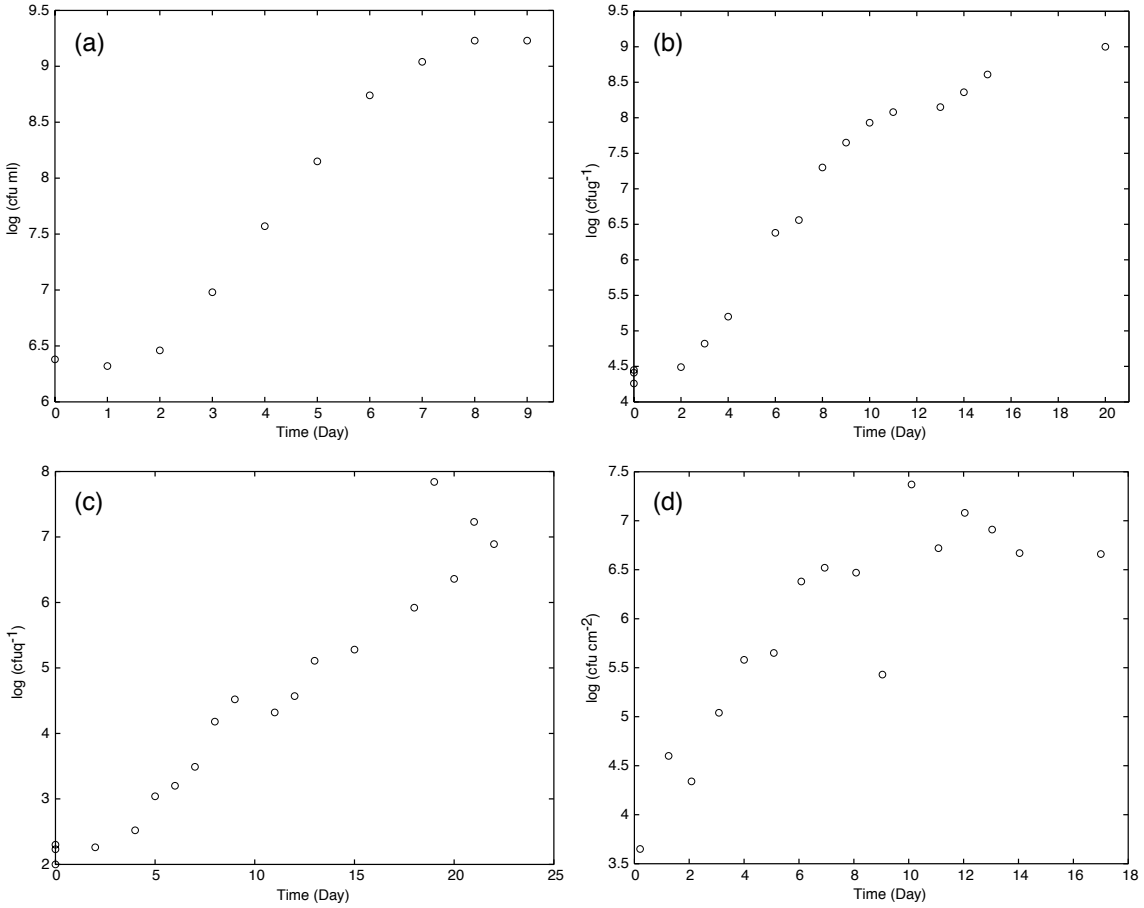

Fig. 1. Typical examples of four growth curves: (a) in broth, (b) and (c) in crushed cold-smoked salmon, (d) on the surface of cold-smoked salmon.

Table 1
Quartiles of the root mean square errors estimated by least-squares regression of the Baranyi and Roberts model to a set of 9 curves in broth, a set of 29 curves in crushed cold-smoked salmon, a set of 24 curves on surface of cold-smoked salmon

Growth environment	Minimum	25th percentile	Median	75th percentile	Maximum
Broth	0.05	0.05	0.07	0.13	0.17
Crushed cold-smoked salmon	0.13	0.19	0.21	0.27	0.43
Surface of cold-smoked salmon	0.11	0.30	0.37	0.43	0.61

ment are observed, the root mean squares errors being definitely smaller for the curves carried out in broth than those obtained on the surface of cold-smoked salmon, the curves carried out in crushed cold-smoked salmon corresponding to an intermediate situation. It can be assumed that values obtained in broth represent mostly the analytical error, due to the viable count procedure. Values obtained for the growth curves in crushed food and even more on food surface are far higher. As the same viable count procedure was applied to all growth curves, we can hypothesise that this difference is explained by additional errors. A first group of errors are measurement/methodological errors or variability: heterogeneity of the initial contamination, and/or heterogeneity of the growth environment between the different packages constituting a growth curve. A second error type is related to the suitability of the Baranyi and Roberts growth model (or any other classical primary model) when real deviations from the exponential growth phase are apparent (for example, slow-downs in the growth, as observed by Cornu et al. (1999) for *Escherichia coli* O157:H7 growing on a minimal liquid medium and Vereecken (2002) for *Lactococcus lactis* on a rich modified BHI medium). Observe that in our case, it was impossible to discriminate from the collected data between a real deviation from the exponential growth, e.g. due to diauxic growth and a deviation due to heterogeneity of the medium. As there is a mixing step at the beginning of a growth curve in crushed salmon and not on the surface of sliced salmon, it is expected that heterogeneity errors are higher in the latter case. The same conclusions can be drawn for the maximal residual of each curve (data not shown). Some of these higher residuals might result from outlier data points (as shown in Fig. 1).

2.2.3. Outliers and normality of residuals

We subsequently focused on the distributions of residuals, which are generally assumed to be Gaussian in non-linear regression. Skewness-values were close to zero (data not shown). Table 2a presents the quartiles of their Kurtosis-values for each of the three growth environments. The Kurtosis-values are far higher than 3 (the reference value for a Gaussian distribution) for some growth curves in crushed salmon and most growth curves on the surface of salmon slices. Such Kurtosis-values may be explained by the non-normality of residuals, and/or by the presence of outliers. Various parametric distributions were fitted to each distribution, and none of these fits could be excluded, as most growth curves have too few data

Table 2
Quartiles of (a) the Kurtosis coefficients of the distributions of residuals of a set of 9 curves in broth, a set of 29 curves in crushed cold-smoked salmon, a set of 24 curves on surface of cold-smoked salmon, and (b) the Kurtosis coefficients of 10 000 simulated Gaussian distributions of 20 points, 10 000 simulated logistic distributions of 20 points, and 10 000 simulated Laplace distributions of 20 points

(a) Growth environment	Minimum	25%	Median	75%	Maximum
Broth	1.66	2.16	2.3	2.97	4.05
Crushed cold-smoked salmon	1.70	2.22	2.78	3.82	5.60
Surface of cold-smoked salmon	2.20	2.78	3.65	5.15	10.60
(b) Simulated distributions	2.5%	25%	Median	75%	97.5%
Gaussian	1.74	2.20	2.54	3.03	4.66
Logistic	1.82	2.42	2.90	3.66	6.45
Laplace	2.06	2.83	3.53	4.60	8.40

points (around 20) and goodness-of-fit tests (Kolmogorov–Smirnoff and Anderson–Darling tests) were not powerful enough. However, on the basis of the null Skewness-values, the symmetrical Gaussian, logistic and Laplace laws appear to be good candidates. A global goodness-of-fit evaluation was then based on simulations. The quartiles of the Kurtosis-values of values sampled from three parametric distributions (Gaussian, logistic, and Laplace) are presented in Table 2b. On the basis of these results, we suppose for the continuation of this work that the residuals of the curves in broth are normally distributed, that those of crushed salmon result from a logistic distribution whereas those of the surface curves result from a Laplace distribution. We cannot exclude other hypotheses, it could be for example hypothesised that residuals could issue from another law, or even a mixture of laws, or that residuals of each growth curve could issue from a Gaussian distribution contaminated by outliers. Further research could focus on the description of these errors, possibly with a continuous adjustment. Nevertheless, our Gaussian/logistic/Laplace hypothesis appears consistent with the observations.

2.2.4. Necessity of a robust alternative to least-squares regression

The main observation of this experimental part is the particularity of growth curves obtained in and on foods, compared to those obtained in broth, with the presence of outliers (see Fig. 1c and d) and/or the non-normality of residuals (see Table 2).

In the case of growth curves in broth, the traditional least-squares criterion is to be preferred since the least-squares estimator is unbiased and has the minimal variance when the residuals are distributed normally. However, in the case of challenge-tests, the least-squares criterion may not be optimal. Indeed, only one outlier can cause a considerable bias of the least-squares estimator, in particular when the number of observations per curve is low, which is generally the case in this field. Potential deficiencies of least-squares methods in such cases have been investigated since the 1960s, and robust estimation was proposed (Huber, 1973; Dutter, 1977). The meaning of "robust" is that a few erroneous observations should not alter the result in a significant way. The purpose is to make a statistical model that is not sensitive to (relatively) gross errors and/or non-normality in the data.

3. Robust regression

From the previous section, it appeared that a robust method may be more adaptive to fit some growth curves obtained in or on cold-smoked salmon to circumvent potential problems related to outliers and/or non-normality of residuals. In this section, robust regression is firstly theoretically presented, secondly validated on the basis of simulated growth curves, and finally applied to experimental growth curves. In this paper, methodological considerations are only shortly introduced, focusing on the selected case-study of primary modelling for challenge tests.

3.1. Robust regression methods

3.1.1. Overview of robust regression methods

There are two robust methodologies developed in order to reduce the impact of outliers (Rousseeuw and Leroy, 1987).

The first approach to using robust methods simply replaces the least-squares objective function (see Eq. (7)), with one less sensitive to outliers, i.e., a robust objective function. In 1887, Edgeworth proposed to replace $\rho(u)=u^2$ by $\rho(u)=|u|$. This defines the l_1 or Least-Absolute Deviation estimator. However, it is difficult to use in non-linear regression due to differentiability issues (Edlund et al., 1997; Rousseeuw and Leroy, 1987). Subsequently, other robust criteria have been proposed. One class of these alternative estimators is the family of M-estimators. The term M-estimator is to be interpreted as a maximum likelihood type estimator, and is justified by the fact that the definition of an M-estimator is somewhat similar to the maximum likelihood problem (Huber, 1981). In this class, the so-called Huber M-estimator is of particular interest in applications, and is presented in the next subsection.

The second approach is to detect outliers in a first step, on the basis of a robust regression. Once identified, outliers are corrected, removed, or down-

weighted, the "good" data are refitted using standard least-squares methods (Shertzer and Prager, 2002). The S-estimators (Rousseeuw and Leroy, 1987), for example the Least-Median of Squares (LMS), are recommended for this first step. A practical problem with the use of LMS is the difficulty of finding the minimum of a LMS objective function, particularly for non-linear optimization methods, where LMS poses a particularly difficult challenge (Stromberg, 1993). Moreover, the correction or removal of suspect data is not always acceptable. In the specific case of our study, outlier data are suspect but cannot be asserted to be aberrant. Potential (large) reporting errors are systematically identified and corrected by food microbiologists and data obtained by incorrect experimental procedures are excluded. To summarize, it is in practice impossible to discriminate whether one or more outlier data represent experimental gross errors (measurement uncertainty) or if they really express unexplained (and unmodelled) biological variability. For these reasons, the LMS method did not appear as suitable for growth curves with suspect data or non-normal errors. This S-estimator will not be investigated further.

3.1.2. The M-estimators

In robust regression, it is necessary to transform the regression model (see Eq. (7)), to obtain invariance of errors by transformation (Huber, 1981). The residual entries are rescaled by a factor σ (standard error of the residuals), giving the following optimization problem:

$$\min_x \sum_{i=1}^{n} \rho\left(\frac{r_i(x)}{\sigma}\right) \tag{8}$$

where $\rho(u) \geq 0$, ρ is a decreasing function when $u<0$ (and an increasing function when $u>0$). The scaling factor σ can be either known (and fixed) or estimated in the same optimization procedure. In the case of growth curves, it appears difficult, or even impossible, to fix a value for σ.

As discussed higher, both measurement and model uncertainties have to be dealt with in the case of growth curves obtained in or on smoked salmon, and the errors are higher then when tested in a liquid medium. In other words, the standard error σ or RMSE as an estimator of σ are data-specific and cannot be fixed beforehand. The least-squares estimator of σ or RMSE might be used but is not robust. Croux and Rousseeuw (1992) proposed to use the S_n estimator defined by:

$$S_n = k_2 \mathrm{med}_i \mathrm{med}_{j \neq i} |r_i - r_j| \tag{9}$$

where med is the median and k_2 is a constant (one per type of residual law). In the case of the Gaussian distribution, the constant k_2 was fixed at the classical value of 1.1926 (Croux and Rousseeuw, 1992). For Logistic and Laplace laws, we used the procedure described by Croux and Rousseeuw (1992) to calculate k_2, and we obtained $k_2 \cong 1.28$ in the first case and $k_2 \cong 1.53$ in the latter case.

3.1.3. The Huber M-estimator

In Eq. (8), various functions ρ can be used. The Huber M-estimator proved to be the M-solution of a contaminated Gaussian error model (Huber, 1973, 1981). It is a widely used robust estimator, defined by:

$$\rho(u) = \begin{cases} \dfrac{u^2}{2} & \text{if } |u| \leq c \\ c|u| - \dfrac{c^2}{2} & \text{if } |u| > c \end{cases} \tag{10}$$

c is a tuning constant and $c=1.345$ by default. When the residuals are normally distributed and the tuning constant is set at the default value, they give the procedure about 95% of the efficiency of classical ordinary least-squares. The Huber estimator coincides with the least-squares estimator as a limiting case when the tuning parameter approaches infinity. Furthermore, if we let c tend to zero, the Huber estimator will approach the l_1-estimator. For intermediate values, the purpose is to treat the majority of data as in least-squares estimation, while possible outliers are limited in their weight in the error function.

3.2. Comparison of least-squares regression and robust regression on the basis of simulated growth curves

After this theoretical introduction, we tested the application of the Huber estimator to identify the

parameters of the Baranyi and Roberts primary model. Simulations are performed to compare both the bias and precision of the Huber and the least-squares estimators concerning their ability to find the known parameters underlying the simulated growth curves. A method is all the more unbiased as the mean of the estimated parameters is close to the fixed known value. A method is all the more precise as the variance of the estimated parameters is close to zero.

3.2.1. Simulation procedure

In the simulation study, "perfect" growth curves were first simulated, using the Baranyi and Roberts primary model with fixed values for each of the four parameters (μ_{max}, y_0, λ and y_{max}). Realistic parameters were chosen: $\bar{x}_1=\mu_{max}=0.8$ day^{-1}, $\bar{x}_2=y_0=1$ log (cfu/cm^2), $\bar{x}_3=\lambda=3$ days and $\bar{x}_4=y_{max}=7$ log (cfu/cm^2).

Subsequently, "realistic" growth curves were simulated, by adding residuals, randomly sampled from various distributions. Each simulated curve had 20 data points, at 20 equidistant sampling times t_k.

The jth simulated curve is then:

$$\bar{y}_{jk} = f(\bar{x}, t_k) + \hat{r}_{jk} \quad (11)$$

for $k=1,\ldots,20$ where $\bar{x}=(\bar{x}_1, \bar{x}_2, \bar{x}_3, \bar{x}_4)$, and $\hat{r}_{jk} \rightsquigarrow D(m=0, \sigma)$ (D being a Gaussian, Logistic or Laplace distribution, with expectation $m=0$, and standard deviation $=\sigma$).

Gaussian, Logistic or Laplace distributions are supposed to represent distributions of residuals respectively in broth, in crushed cold-smoked salmon, and on the surface of the salmon (as based on the results presented in Section 2.2.3). Two different values of σ were tested per distribution type: the minimal and the maximal root mean squares errors in each growth environment. For the Gaussian law (which simulated the curves in broth), $\sigma=0.05$ and 0.17; for the Logistic law (which simulated the curves in crushed cold-smoked salmon), $\sigma=0.13$ and 0.43; for the Laplace (which simulated the curves on cold-smoked salmon), $\sigma=0.11$ and 0.61.

For each distribution, 5000 20-point curves were simulated. The four parameters of the Baranyi and Roberts model were estimated for each simulated curves, both by least-squares regression, and by robust regression, using the Huber M-estimator. The least-squares regression method was similar to the one defined in Section 2.1.3. The robust regression method required the procedure fmincon of the Matlab Optimization Toolbox, making use of a Sequential Quadratic Programming method with an incorporated Quasi-Newton updating procedure. The S_n estimator was calculated as defined in Section 3.1.2.

The means and variances of the 5.000 sets of parameters for each distribution were compared to assess the bias and precision of these estimators.

3.2.2. Simulation results

Table 3 presents the estimated bias and precision of each regression method, in six different cases.

The Gaussian errors were supposed to simulate growth curves in broth. In this case, and in the absence of outliers, it is well-known that the least-squares estimator is optimal and has to be used. For both tested values of σ and for the four parameters, the least-squares estimator appears indeed less biased and more precise than the Huber M-estimator (Table 3a and b).

With the Logistic distributions, which were supposed to simulate growth curves in crushed cold-smoked salmon, the least-squares estimator also appears less biased (except for one parameter, the lag time) and more precise (for all parameters) than the Huber estimator (Table 3c and d), even if the difference between the two methods is less pronounced than with the Gaussian distributions.

On the contrary, with the Laplace distributions, which were supposed to simulate growth curves on surface of salmon, the robust estimator appear less biased (except for the lag time and the maximal population in the last simulation), and more precise (for all parameters in both simulations) than the least-squares regression (Table 3e and f).

The use of the Huber M-estimator is then to be recommended in the case of such non-Gaussian errors, which may be encountered in the case of challenge tests on surface of foods.

Last, it has to be underlined that these simulations were based on pure theoretical distributions, not contaminated with outliers. As mentioned earlier, it is a known result that the robust regression is also more adequate in the presence of outliers.

Table 3
Bias and precision of the least-squares (LS) and robust (Huber) estimators

	μ_{max} (theoretical value: 0.8 day^{-1})		y_0 (theoretical value: 1 log cfu/g)		λ (theoretical value: 3 days)		y_{max} (theoretical value: 7 log cfu/g)	
	LS	Huber	LS	Huber	LS	Huber	LS	Huber
(a) Gaussian (0,0.05)								
Mean (Bias)	0.8003 (+0.04%)	0.8004 (+0.05%)	1.0001 (+0.01%)	1.0002 (+0.02%)	3.0030 (+0.1%)	3.0024 (+0.08%)	7.0004 (+0.01%)	7.0009 (+0.01%)
Variance (CV)	0.00007 (1%)	0.00009 (1.2%)	0.0013 (3.6%)	0.0015 (3.9%)	0.0279 (5.6%)	0.0327 (6%)	0.0011 (0.5%)	0.0013 (0.5%)
(b) Gaussian (0,0.17)								
Mean (Bias)	0.8018 (+0.23%)	0.8025 (+0.31%)	0.9975 (−0.25%)	0.9963 (−0.37%)	2.9955 (−0.15%)	2.991 (−0.3%)	7.0086 (+0.12%)	7.0137 (+0.2%)
Variance (CV)	0.0008 (3.5%)	0.0011 (4.1%)	0.0146 (12.1%)	0.0198 (14.1%)	0.3176 (18.9%)	0.4259 (21.8%)	0.0148 (1.7%)	0.021 (2%)
(c) Logistic (0,0.13)								
Mean (Bias)	0.8009 (0.11%)	0.8012 (0.15%)	0.9963 (−0.37%)	0.9971 (−0.29%)	2.9904 (−0.32%)	2.9939 (−0.2%)	7.0051 (+0.07%)	7.0054 (+0.08%)
Variance (CV)	0.0004 (2.5%)	0.0005 (2.8%)	0.0084 (9.2%)	0.0105 (10.3%)	0.176 (14%)	0.2178 (15.6%)	0.0088 (1.3%)	0.0107 (1.5%)
(d) Logistic (0,0.43)								
Mean (Bias)	0.8130 (+1.62%)	0.8131 (+1.64%)	0.9722 (−2.78%)	0.9713 (−2.87%)	2.9596 (−1.44%)	2.967 (−1.10%)	7.0965 (+1.38%)	7.1001 (+1.43%)
Variance (CV)	0.0056 (9.4%)	0.0058 (9.5%)	0.0963 (31.0%)	0.101 (31.8%)	2.1088 (48.0%)	1.9622 (46.7%)	0.2859 (7.6%)	0.3168 (8.0%)
(e) Laplace (0,0.11)								
Mean (Bias)	0.8006 (+0.07%)	0.8004 (+0.05%)	0.9976 (−0.24%)	0.9979 (−0.21%)	2.9887 (−0.38%)	2.9932 (−0.23%)	7.0026 (+0.04%)	7.0025 (+0.04%)
Variance (CV)	0.00032 (2.2%)	0.00027 (2.1%)	0.0059 (7.7%)	0.0057 (7.5%)	0.1169 (11.4%)	0.1216 (11.6%)	0.0062 (1.1%)	0.0060 (1.1%)
(f) Laplace (0,0.61)								
Mean (Bias)	0.8342 (+4.28%)	0.8277 (+3.46%)	0.9706 (−2.94%)	0.9679 (−3.21%)	3.0726 (+2.42%)	3.0248 (+0.83%)	7.1830 (+2.61%)	7.1855 (2.65%)
Variance (CV)	0.0196 (17.5%)	0.0157 (15.7%)	0.1714 (41.4%)	0.1520 (39%)	3.5990 (63.2%)	3.1249 (58.9%)	0.6054 (11.1%)	0.5887 (11.0%)

Means, bias=(mean − theoretical value)/theoretical value (%), variances and coefficients of variation=standard deviation/theoretical value (%) of parameters estimated by LS and Huber regression by fitting realistic simulated growth curves, using the Baranyi and Roberts primary model. Realistic growth curves were obtained with the indicated theoretical values for the four parameters by (a) adding Gaussian residuals (mean=0 and standard error=0.05), (b) adding Gaussian residuals (mean=0 and standard error=0.17), (c) adding Logistic residuals (mean=0 and standard error=0.13), (d) adding Logistic residuals (mean=0 and standard error=0.43), (e) adding Laplace residuals (mean=0 and standard error=0.11), (f) adding Laplace residuals (mean=0 and standard error=0.61).

3.3. Comparison of least-squares regression and robust regression on the basis of real growth curves

In this last step, Huber and least-squares estimators were compared on the basis of three representative growth curves (in crushed cold-smoked salmon or on the surface of cold-smoked salmon slices) previously presented in Fig. 1.

3.3.1. Regression procedure

Both methods were used to fit the experimental growth curves, as described in Section 3.2.1. In the presence of an (apparent) outlier in a curve, the adjustment was carried out twice by both methods, firstly with all data points, and secondly after deleting this outlier.

3.3.2. Regression results

Three typical examples are presented (Figs. 2–4). These growth curves were carried out in crushed cold-smoked salmon (Figs. 2 and 3) and on the surface of cold-smoked salmon slices (Fig. 4). One of the crushed salmon curves (Fig. 2) is in conformity with the theoretical model (which was the case of the majority of the curves carried out in this medium) whereas the second one (Fig. 3) presents an outlier. The surface curve (Fig. 4) presents also a potentially aberrant point (at day 9), and large residual values for most data points, which was representative of most surface curves. For the first growth curve in crushed cold-smoked salmon (Fig. 2), the Huber-estimator is clearly less influenced by the last, higher point. For an ideal curve, it is preferable to use standard least-

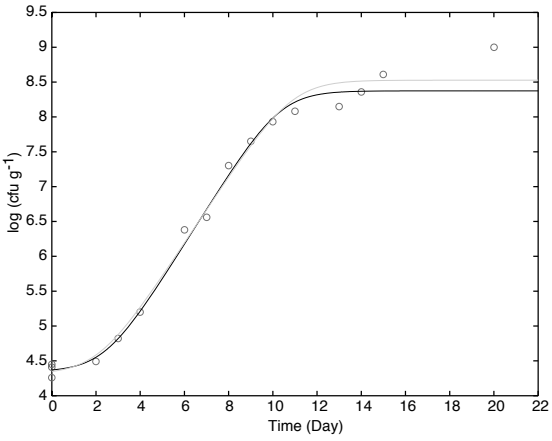

Fig. 2. Growth curve in crushed cold-smoked salmon (see Fig. 1b): Experimental data points (○), result of the least-squares regression: fitted Baranyi model (–), result of the robust regression: fitted Baranyi model (–).

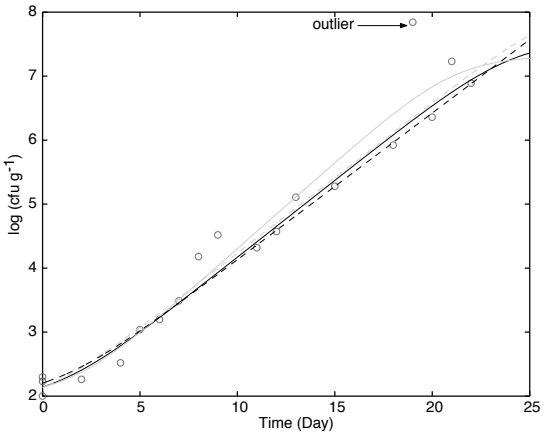

Fig. 3. Growth curve in crushed cold-smoked salmon (see Fig. 1c): Experimental data points (○), result of the least-squares regression: fitted Baranyi model (–), result of the robust regression: fitted Baranyi model (–), Result of the least-squares regression without the outlier: fitted Baranyi model (==), result of the robust regression without the outlier: fitted Baranyi model (– –).

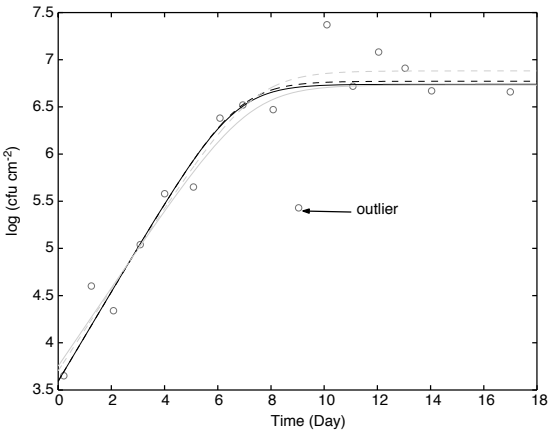

Fig. 4. Growth curve on the surface of cold-smoked salmon (see Fig. 1d): Experimental data points (○), result of the least-squares regression: fitted Baranyi model (–), result of the robust regression: fitted Baranyi model (–), result of the least-squares regression without the outlier: fitted Baranyi model (==), result of the robust regression without the outlier: fitted Baranyi model (– –).

squares regression. Both methods are not equivalent, however they lead to close results.

In the presence of an outlier (Figs. 3 and 4), differences between both fits are more pronounced. It is obvious that the robust regression is less influenced by the outlier, as fits without or with the outlier are much closer with the Huber criterion than with the least-squares criterion. Then, the Huber robust regression appears more adapted in this case.

4. Discussion and recommendations

4.1. Reflections on the assumptions and related realism underlying challenge tests

Challenge testing is a key element in model validation. Indeed, whatever their construction (based on challenge tests, growth curves in broth, or both), predictive models must be shown to predict accurately the behaviour of micro-organisms in real foods or during real food processes. This involves comparison of predicted responses to observations in products and/or processes, independent of those used to generate the model (Ross, 1996). Criteria have been proposed for a quantitative validation (Ross, 1996; Baranyi et al., 1999).

When validating broth-based models to predict growth of the spoilage flora in foods, Pin et al. (1999) introduced three levels of errors. They aimed to reflect the increase in complexity of the system, from broth to artificially contaminated foods, and from artificially contaminated foods to naturally spoiled foods.

Firstly, the *primary error* was defined as the difference between growth rates predicted by the model (to be validated) and growth rates estimated from growth curves in broth (either used to create the model, or obtained in similar conditions). As stated by the authors, this error includes the uncertainty associated with the adjustment of the secondary model. It also includes the uncertainty associated with the adjustment of the primary model to obtain the estimated growth rates, which is not negligible, especially in the case of models based on challenge tests.

Secondly, Pin et al. (1999) considered the difference between growth rates predicted by the model and growth rates estimated in artificially contaminated foods. It was named the *intermediate error*. As stated by the authors, this error includes the primary error, and (when the model is broth based) the influence of the growth substrate. Indeed, the structure of the food can affect microbial distribution and growth, due to structural features of the aqueous phase relevant to the microbial length scale (e.g. globules of milk fat in hard cheese, droplets of aqueous phase within an outer oil phase in margarine), see Brocklehurst (2003). This intermediate error also includes the uncertainty associated with the adjustment of a primary model to obtain the estimated growth rates in the inoculated food products.

As Pin and co-authors considered the total spoilage flora, the artificially contaminated foods were sterile foods inoculated by a controlled spoilage flora. The authors of the present research want to stress that when the micro-organism of interest is a pathogen, two types of challenge tests can be considered: either in sterile food products (or with a relatively low spoilage level), or in competition with a naturally occurring spoilage flora. In the latter case, the intermediate error would also include the effect of the microbial interactions, such as the Jameson effect (Ross et al., 2000). Additional errors are certainly to be considered at this step and may be different from one study to another.

Thirdly, the *overall error* was defined by Pin et al. (1999) as the error between predicted growth rates and growth rates estimated in naturally spoiled foods (storage trials). The overall error includes (i) the intermediate error, (ii) the errors due to the difference between artificial and natural contamination, which is discussed below, and (iii) the uncertainty associated with the estimation of growth rates in storage trials, which is a particularly complex question, not further discussed in this paper.

The difference between artificial and natural contamination is indeed an important issue. Even if every effort is made to mimic realistic conditions (see recommendations published in AFSSA, 2001; IFT, 2001; ICMSF, 2002; Brocklehurst, 2003), experimental bias may occur. The inoculated strains are not always representative of the within-species variability; non-pathogenic surrogates have even to be chosen when the inoculation is performed in the food production or processing environment. The level of con-

tamination is usually unrealistically high, to allow efficient plate-counting. Even in the presence of the naturally occurring spoilage flora (non sterile foods), the effect of microbial interactions between the studied micro-organism and other flora is then underestimated. The physiological state of the micro-organism (stress in the environment versus inoculum preparation in the lab) has also to be considered even if it is more crucial for the lag time than for the growth rate. Last, the structure of the food and its a_w might be modified by the inoculation procedure and the preparation of the food samples. Whereas naturally contaminated foods are typically not homogeneous, and not homogeneously contaminated by the micro-organism of interest, homogeneity of both the contamination and the contaminated food should be obtained so that the results of challenge tests are exploitable. A compromise between realism and the potential for data analysis should be reached.

The experimental choice between inoculation onto surfaces or into the food is a crucial question, from this point of view. Surface inoculation (by dip inoculation, spray inoculation or spot inoculation) has been widely used: on meat products, including wieners, frankfurters and sausages (McKellar et al., 1994; Islam et al., 2002a; Samelis et al., 2002; Taormina and Beuchat, 2002; Naim et al., 2003); cooked ham and pâté (Uyttendaele et al., 2004), sliced cooked cured pork shoulder (Mataragas et al., 2003), chicken luncheon meat (Islam et al., 2002b), and chicken breast mince and chicken thigh burgers (Oscar, 2002); on cheeses (Jensen and Knöchel, 1995; Stecchini et al., 1995; Eppert et al., 1997; Loessner et al., 2003); and on vegetables, including freshly peeled oranges (Pao and Brown, 1998), and external surfaces (rind) of cantaloupe (Ukuku and Fett, 2002). A major interest of surface inoculation is to gain realism, as some sources of contamination lead to surface contaminations. Moreover, it reduces the modification of the structure of the food. However, such an inoculation, which excludes any mixing step, appeared in the present study as an important contribution to the difficulty of exploiting challenge tests, as more (apparent) outliers and higher RMSE values are obtained in comparison with crushed food, and even more in comparison with broth-based data. Once again, a compromise has to be reached between realism and interpretability of a challenge test.

4.2. Recommendations for fitting growth curves obtained in and on food products

One of the main objectives of challenge testing is to estimate growth parameters (especially the growth rate), either to build a new secondary model or to validate an existing one. Recommendations for this step were rarely published, as far as we know.

Needless to say that careful use of non-linear regression methods and software packages is an important prerequisite. On the contrary, simple calculations based on the difference between population densities at two distant sampling times, which are still practiced (AFSSA, 2001; FDA, 2003), are far less precise and robust.

Among least-squares regression methods, the most common choice is the least-squares regression, which is implemented in all classical optimization/statistical software. The least-squares solution is the maximum likelihood solution if the errors are normally distributed, but it is not very good in handling erroneous observations, since there is a high penalty on large residual entries. As demonstrated in Sections 2 and 3, the least-squares criterion can appear ineffective for certain sets of data. Indeed, for some growth curves (at least on the surface of cold-smoked salmon, as shown in Section 2), the errors can deviate from the assumption of normality. In the same way, certain points of the experimental curves appear suspect and may reflect measurement or model uncertainty increasing when going from broth, to crushed salmon and to the surface of cold-smoked salmon slices. However, it would be incorrect to delete these potentially aberrant points from the analysis without any statistical justification. If the previous experimental recommendations are carefully followed, this problem might be minimized (but not systematically avoided). For these reasons, we recommend the use of robust non-linear regression to model challenge tests in two important (and potentially related) cases: when the inoculated samples are potentially heterogeneous and heterogeneously contaminated (typically the case of surface inoculation), and when the observation of the growth curve reveals outliers. There seems to be a large correspondence between the particular features of challenge tests (when compared to standard growth curves in broth) and the optimal conditions of

robust regression (when compared to standard least-squares regression). Robust regression has to be implemented using an appropriate software package. In this study, it was implemented using Matlab, and an example of a suitable Matlab-coding is available upon request from the authors.

Despite all these precautions, it has to be clearly stated that the estimation of a growth rate from data obtained in a challenge test is never a perfectly precise determination, but in most cases a gross estimation. For this reason, we highly recommend giving the confidence interval on each estimated growth parameter. Such confidence intervals can be obtained by different methods, developed in the field of non-linear regression (Huet et al., 1992, 1996; Van Impe et al., 2001): standard errors (only approximative for non-linear models), joint confidence regions, likelihood methods. Resampling methods are also available: Jackknife (Baty and Delignette-Muller, 2004), and Bootstrap (Huet et al., 1992). Last, Monte Carlo simulations based on the experimental error were proposed by Poschet et al. (2003).

A promising way to decrease this estimation uncertainty could be to use larger data sets. Whereas it is of most common practice in food microbiology to consider each growth curve separately, as it was done in the present study, alternative approaches in which several growth (or inactivation) curves are fitted simultaneously have been proposed. These approaches usually rely on the use of a global model, made of a primary and a secondary model, with fixed effects (Bernaerts et al., 2001, 2002, Fernandez et al., 2002; Skandamis et al., 2002; Valdramidis et al., 2005), random effects (Juneja and Marks, 2002; Montañez et al., 2002; McCann et al., 2003; Shorten et al., 2004), or hyperparameters in a Bayesian approach (Pouillot et al., 2003). These approaches appear promising in the case of challenge tests, as they may allow for much better overall estimation.

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Effect of the inoculum size on *Listeria monocytogenes* growth in structured media

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Abstract

Obtaining quantitative data concerning the relative impact of various factors that may influence bacterial growth is of great importance for microbial risk assessment and predictive microbiology. The objective of this work was to investigate the effect of the initial *Listeria monocytogenes* density on all the growth parameters of this pathogen (lag phase duration, growth rate and maximum population density attained) on a sterile solid model system mimicking smoked fishery products, and in real cold-smoked salmon, a product likely to be contaminated with *L. monocytogenes*. Growth of the pathogen was monitored using a sensitive enumeration method, recently developed, based on membrane filtration followed by the transfer of the filter on a selective media [Gnanou Besse, N., Audinet, N., Beaufort, A., Colin, P., Cornu, M. and Lombard, B., 2004. A contribution to the improvement of *Listeria monocytogenes* enumeration in smoked salmon. International Journal of Food Microbiology, 91, 119–127.]. Depending on the experimental conditions, we found a significant effect of the inoculum size, both on lag phase duration, and on the maximal population attained. Moreover, the effect of the inoculum size on the growth of *L. monocytogenes* was dependent on a complex set of interactions. Factors which have appeared to impact on this effect include the cells physiological state, the background microflora, the texture of the media and the packaging system. It is important to understand how these interactions affect the growth of *Listeria* in order to predict and control its development in food.

Keywords: *Listeria monocytogenes*; Kinetics; Inoculum size; Fishery products

1. Introduction

Listeria monocytogenes is a Gram-positive bacterium responsible for listeriosis, a severe foodborne illness which may result in meningitis, septicemia, spontaneous abortion, perinatal infections and gastroenteritis. Despite the low incidence of infection, listeriosis is associated with a high rate of lethality, particularly in elderly and immuno-compromised individuals (Anonymous, 2000). Detection of *L. monocytogenes* in a food product may also have important economic consequences for a manufacturer, resulting from the recall and withdrawal of contaminated products, as well as decreased sales for incriminated food products.

Getting quantitative data concerning the relative impact of various factors that may influence bacterial growth is of great importance for predictive microbiology and microbial risk assessment. In the case of *L. monocytogenes*, this gave place to a considerable amount of research tasks, having led to the identification of numerous environmental variables and of factors linked to the initial physiological state of the population and to the initial density of the pathogen. Schematically, a typical microbial curve has three distinct phases: (i) an initial lag phase, during which the N_0 microbial cells adapt to their new environment, (ii) an exponential growth phase, where cells are multiplying at a certain rate, μ, (iii) and a stationary phase, where the maximum population density N_{max} is reached and growth ceases.

Some studies realized under laboratory growth conditions and also in food suggested that the inoculum size had no effect

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on subsequent *L. monocytogenes* growth (Buchanan and Philips, 1990; Wimpfheimer et al., 1990; Lanciotti et al., 1992; Duffy et al., 1994; Brouillaud-Delattre et al., 1997). However, more recently, an effect of the inoculum size on the lag phase duration has been observed in sterile synthetic broth, under particular stress or suboptimal conditions, when the inoculum was small (Gay et al., 1996; Augustin et al., 2000; Robinson et al., 2001). This increase of the lag phase at the low inoculum density can be explained by the statistical effect arising from the lag phase variability of individual cells (also called stochastic effect, Baranyi, 2002). This phenomenon is emphasized under suboptimal or stress conditions since in these cases, lag phase is getting longer and distribution of individual lag phase is getting broader (Baranyi and Pin, 1999; Robinson et al., 2001; Baranyi, 2002; Francois et al., 2002; Metris et al., 2003). The distribution of the individual *Listeria* lag times have been analyzed, linked by mathematical formula to the lag phase of the total population, and taken into account in *Listeria* growth models (Baranyi and Pin, 1999; Mc Kellar and Knight, 2000; Baranyi, 2002). A possible effect of cell–cell interaction on bacterial growth has also been suggested by Kaprelyants and Kell (1996), on the basis of personal observations and of a bibliographic review. Communication between cells is not well documented for *L. monocytogenes*; however some studies have suggested the possibility of density dependant patterns, that may impact on the lag phase duration (Robinson et al., 2001). Studies which examined the effect of the inoculum size on the lag of *L. monocytogenes* had to face technical limitations, one of the most important being the lack of sensitivity of standard methods such as the reference method EN ISO 11290-2 (Anonymous, 1998a) for the enumeration of *L. monocytogenes* in food. From a practical point of view, it is consequently necessary to use quite high inoculum size, which do not reflect the low contamination levels usually found in food (Anonymous, 1998b), to extrapolate growth curves or to use other enumeration techniques such as the Most Probable Number (MPN) method, which do not provide accurate results (Oblinger and Koburger, 1975). In some cases, the observation of the inoculum size effect on the lag phase duration could not be confirmed by the statistical analysis, because of the large variability and high standard error associated with the results (Duffy et al., 1994).

By contrast, it is generally assumed that the bacterial specific growth rate is independent of the inoculum size. However, Gay et al. (1996) and Coleman et al. (2003) observed an unexpected density dependence of respectively *L. monocytogenes* and *Escherichia coli* growth rates in broth, under suboptimal conditions. In this last study, a "quorum sensing" effect was suggested.

A correlation between the *L. monocytogenes* inoculum size and the maximal population attained in various foods has also been reported (Guyer and Jemmi, 1991; Peterson et al., 1993; Pelroy et al., 1994; Carlin et al., 1995) and was, in most cases, attributed to interactions with the food background microflora or to a greater impact of food inhibitors at low inoculum level. To date, there is, to our knowledge, no study which has examined the effect of the inoculum size on the maximal population attained in solid sterile media. Despite the fact that foods are often of solid consistency, experiments carried out in order to study the inoculum size effect on the growth of *L. monocytogenes* were generally carried out in liquid media, where cells display a planktonic growth pattern. Growth pattern of immobilized cells into colonies is of great interest in the field of food microbiology, and is likely to differ from dispersed growth in a liquid medium. Moreover, most of growth studies in real food concerned maximal population attained, without elaboration of complete growth curves.

The objective of this work is to investigate the effect of the inoculum size on growth parameters of *L. monocytogenes* (lag phase duration, growth rate and maximum population density) on a sterile solid media model system mimicking smoked fishery products and by comparison in cold-smoked salmon, a product likely to be contaminated with *L. monocytogenes* (Anonymous, 1999). The relative impact of various physiological and environmental factors (stress of the inoculum, food texture, packaging, background microflora) that could interact with the inoculum size and influence *L. monocytogenes* growth will then be estimated. *L. monocytogenes* growth was followed using a newly developed sensitive enumeration method based on membrane filtration (Gnanou Besse et al., 2004), thus allowing to reach very low inoculum densities in solid media.

2. Materials and methods

2.1. Strains and culture conditions

2.1.1. Bacterial strains and preparation of inocula

Experiments were carried out with one strain of *L. monocytogenes* serovar 1/2a (strain S1, Afssa collection), isolated from cold-smoked salmon and characterised in our laboratory. A stock culture was maintained frozen at −80 °C using Cryobank tubes (AES Laboratoires, Combourg, France). Unstressed inocula were prepared as follows: Cultures were revived by plating onto Tryptone Soya with Yeast Extract Agar (TSAYE, AES Laboratoires) and then propagated twice (6 and 18 h cultures at 37 °C) in Brain Heart Infusion broth (BHI, AES Laboratoires) before use. The final BHI culture contained approximately 10^9 CFU ml^{-1}. All dilutions were prepared in Tryptone Salt (TS, AES Laboratoires).

Two successive cultures of 24 h and 4 weeks at 37 °C in BHI served as nutritionally injured inocula. After 4 weeks, a decrease in cell viability ranging from 1.7 to 2 $\log_{10}$ CFU ml^{-1} and an injured cells percentage ranging from 50% to 51% were observed for all inocula used. The percentage of injured cells was determined by differential counts on selective Palcam agar (AES Laboratoires) and on non-selective TSAYE agar. Before use, injured inocula were washed three times in TS by centrifugation (13000 rpm, 2 min).

2.1.2. Media

We used two types of solid media model systems, which differed regarding the solidifying agent. The agar medium contained 15 g l^{-1} agar (AES Laboratoires), whereas the gelatin medium contained 120 g l^{-1} gelatin (melting point 28 °C, 104070, Merck). The gelatin medium is first gently stirred at 50–60 °C, in order to ensure the entire dissolution of the gelatin,

then the medium is sterilized for 10 min at 115 °C. Model systems simulated cold-smoked salmon, with NaCl, pH, phenols values that may correspond to this type of product (Leroi et al., 2001). Both media contained 30 g l^{-1} Tryptone Soya (AES Laboratories), 6 g l^{-1} Yeast Extracts (AES Laboratories) and 40 g l^{-1} NaCl (Merck). Liquid smoke (Lutetia L1165, kindly supplied by Lutetia, Arnouville Les Gonesse, France) was added in order to reach 6 ppm phenols. This liquid smoke was chosen because of organoleptic characteristics very similar to cold-smoked salmon (IFREMER and Lutetia, personal communication). pH was adjusted to 6.1–6.3 with 1 M HCl. Twenty milliliters of the media were poured into 9 cm diameter Petri dishes, left to solidify and kept at 4 °C before use. For artificial contamination, 0.1 ml of appropriate TS dilution of the inoculum was spread out on the surface of the plates.

Cold-smoked salmon was collected from two different French manufacturers A and B. They were purchased and used at the beginning of their shelf-life. Cold-smoked salmons A and B mainly differed regarding their background microflora levels, which were quite constant according to the origin of the product: the aerobic mesophilic microflora level ranged from <10 CFU g^{-1} for salmon A to 10^4–10^5 CFU g^{-1} for salmon B. Absence of *Listeria* spp. in each purchased cold-smoked salmon was previously checked according to the ISO 11290-1 reference method (Anonymous, 1996). Results showed that no product used in these studies was contaminated by *Listeria* spp. Depending on the experimental conditions, cold-smoked salmon of a same batch were either cut into 2 cm square parts and divided into 20 g portions, taking squares from different parts of the salmon sample, or crushed, carefully mixed and divided into 10 g portions. Square slices were used to better simulate growth on the surface of the product. Portions were stored at 2 °C in oxygen-impermeable film bags (PAPE 90, Euralpack, Saint Pierre du Perray, France) before use. For artificial contaminations, each portion was spiked with 0,1 ml of the appropriate TS dilution of the *L. monocytogenes* culture, and thoroughly mixed (crushed products) or gently spread (square slices).

2.1.3. Growth conditions

A growth temperature of 8 °C was selected to simulate the storage temperature of cold-smoked salmons. Samples were incubated under aerobic or anaerobic conditions. Anaerobic conditions were achieved using vacuum-sealed impermeable film packages (Sealboy 235, Multivac type A 300/16 and PAPE 90, Euralpack). In this case, synthetic media were transferred with a spoon from the Petri dishes to the oxygen-impermeable bags. Thus only the media, without the dishes, were incubated.

2.2. Microbiological analysis and experimental design

2.2.1. Microbiological analysis

Periodically, one sample was removed for microbiological analysis. Growth of *L. monocytogenes* was monitored using two types of enumeration methods. In the case of high contamination levels, the EN ISO 11290-2 reference method for enumeration of *L. monocytogenes* (Anonymous, 1998b) was used. In the case of low contamination levels, we used a more sensitive enumeration method, recently developed, based on membrane filtration followed by the transfer of the filter on a selective media (Gnanou Besse et al., 2004). Briefly samples were homogenised in TS diluent (1 in 10 dilution). Increasing volumes (up to 30 ml) of suspension were immediately treated for 20 min at 37 °C in a water bath shaker with 0.83% Tween 80 (P 4780, Sigma-Aldrich, Saint Quentin Fallavier, France) and 0.83% trypsin 1/250 (Difco 0152-13-1), and filtered. The filters were laid onto selective medium, and the plates were incubated upside down at 37 °C for 48 h. In this case, for the gelatine medium, the TS diluent was prewarmed to 37 °C in order to easily dilute and melt the medium, and the enzymatic treatment recommended to improve the filterability of the suspensions was not necessary. In both cases, the selective medium used was Aloa agar (AES Laboratories).

In the case of cold-smoked salmon experiments, growth of the aerobic mesophilic microflora was monitored in parallel, on non-inoculated samples, using the NF V 08-051 method (Anonymous, 1992).

2.2.2. Experimental design

Experiments were designed to meet three main purposes:

(i) to assess the influence of the stress on the inoculum size effect on the growth of *L. monocytogenes*: gelatine media was inoculated with stressed or unstressed inoculum, at concentrations ranging from 10^{-1} to 10^5 CFU g^{-1} and incubated aerobically; sliced or crushed cold-smoked salmon A was inoculated with injured or uninjured cells at concentrations ranging from 10^{-1} to 10^2 CFU g^{-1} and vacuum packed;

(ii) to determine the impact of the medium texture combined with the packaging method on the effect of the inoculum size on the growth of *L. monocytogenes*: gelatine media or agar media were inoculated with uninjured cells at concentrations ranging from 10^{-1} to 10^3 CFU g^{-1}, and incubated aerobically or under vacuum packaging; sliced or crushed cold salmon A was inoculated with uninjured cells at concentrations ranging from 10^{-1} to 10^2 CFU g^{-1} and vacuum packed. By comparison, an aerobic incubation was also performed only on sliced samples, which were more representative of the real texture of cold-smoked salmon;

(iii) to roughly evaluate the effect of different background microflora levels on the inoculum size effect on the growth of *L. monocytogenes*: crushed cold-smoked salmons A and B were inoculated with uninjured inoculum at concentrations ranging from 10^{-1} to 10^2 CFU g^{-1} and vacuum packed. The growth of the aerobic mesophilic microflora was monitored in parallel on non-inoculated crushed samples of cold-smoked salmon A and B.

For practical reasons, most of the growth on model system were performed in aerobic conditions. Experiments in cold-smoked salmon were performed using more realistic conditions: contamination levels <10^2 CFU g^{-1}, mainly anaerobic incubation. A unique salmon batch was used for all the curves of an experiment. In order to obtain curves with weaker standard errors,

Table 1
Conditions, fitted growth parameters (N_0, lag, μ and N_{max}), with 95% asymptotic confidence intervals, and root mean square error (RMSE) for each growth curve of *L. monocytogenes* in synthetic (gelatine-based or agar-based) solid media, at 8 °C

Figures number[a]	Medium	Vacuum	Stress	N_0	Lag	μ	N_{max}	RMSE
1a	Agar	No	No	−0.6 [−1,−0.2][b]	3.0 [1.6,4.4]	1.1 [0.9,1.3]	5.9 [5.3,6.5]	0.40
1a	Agar	No	No	1.3 [0.7,1.8]	2.1 [0.5,3.8]	1.0 [0.9,1.2]	8.6 [7.9,9.3]	0.45
1a	Agar	No	No	3.4 [3.1,3.8]	3.2 [1.9,4.5]	1.2 [0.9,1.4]	9.0 [8.6,9.4]	0.39
1b	Agar	Yes	No	−0.7 [−1.4,0]	1.9 [0.4,3.4]	1.3 [1.2,1.4]	8.8 [8.0,9.6]	0.47
1b	Agar	Yes	No	1.6 [1.3,1.9]	2.5 [1.8,3.1]	1.3 [1.2,1.4]	8.6 [8.4,8.8]	0.17
1b	Agar	Yes	No	3.5 [3.4,3.7]	2.5 [2.1,2.9]	1.4 [1.3,1.5]	8.9 [8.7,9.0]	0.14
1c	Gelatine	No	No	−0.5 [−1.8,0.8]	0.6 [0,2.6]	1.8 [1.4,2.2]	6.4 [5.9,6.9]	0.59
1c	Gelatine	No	No	1.5 [0,3]	0.3 [−3.1,3.6]	1.3 [0.9,1.8]	8.0 [7.4,8.6]	0.68
1d	Gelatine	Yes	No	−0.6 [−1.4,0.3]	1.6 [−0.7,4.0]	1.1 [0.9,1.3]	6.1 [5.4,6.9]	0.55
1d	Gelatine	Yes	No	1.5 [0.2,2.7]	0.5 [0,3.9]	1.0 [0.8,1.3]	8.0 [7.3,8.7]	0.58
1e	Gelatine	No	Yes	−1.0 [−1.3,−0.7]	4.6 [3.3,5.8]	1.2 [0.8,1.6]	3.5 [2.7,4.3]	0.59
1e	Gelatine	No	Yes	3.0 [2.5,3.4]	2.2 [0.9,3.5]	1.1 [0.9,1.3]	8.6 [7.7,9.5]	0.36
1e	Gelatine	No	Yes	5.3 [5.1,5.5]	1.9 [1.4,2.5]	1.3 [1.0,1.5]	8.4 [8.1,8.7]	0.14
1f	Gelatine	No	Yes	−0.1 [−0.6,0.3]	2.7 [1.2,4.2]	1.0 [0.7,1.3]	3.9 [1.5,6.3]	0.42
1f	Gelatine	No	Yes	0.9 [0.4,1.3]	2.9 [1.6,4.2]	1.2 [0.9,1.5]	5.2 [3.8,6.6]	0.42
1f	Gelatine	No	Yes	2.9 [2.5,3.3]	2.3 [1.2,3.5]	1.3 [1.1,1.5]	>7.8	0.38

Estimations were obtained using the logistic model with delay.
[a] Figures number indicates in which figures the curves indicated in the table are represented.
[b] Data in brackets represent 95% asymptotic confidence intervals.

most of the experiments in cold-smoked salmon were performed using crushed products, as recommended by Miconnet et al. (2005). For a given medium and growth condition, assays with different inoculum size to be compared were conducted in parallel. All the curves presented in the same graph were conducted in parallel.

2.3. Curve fitting and statistical analysis

Growth curves were fitted to the Baranyi model (Baranyi and Roberts, 1994) and to the logistic equation with delay (Kono, 1968). Fits were performed by minimizing the sum of squared errors between the data points and the model prediction. In order to estimate the quality of the fit of both models, least square means were compared. On this basis, the logistic model with delay was finally selected. Four parameters (initial population level, lag, growth rate: μ, and maximal population attained: N_{max}) and their standard error were estimated by the model, and compared.

Eventually, the hypothesis that in each particular experiment, the *L. monocytogenes* culture had the same growth parameters whatever the inoculum size was tested in some cases, by performing an F test based on the likelihood ratio (Bates and Watts, 1988; Cornu et al., 1999). A model common to the growth curves obtained in one experiment with different inoculum sizes was fitted. The equality of growth parameters (lag, μ, N max) was tested by comparing a full model (growth parameters different for each curve) to a partial model (with the constrains

Table 2
Conditions, fitted growth parameters (N_0, lag, μ and N_{max}), with 95% asymptotic confidence intervals, and root mean square error (RMSE) for each growth curve of *L. monocytogenes* in cold-smoked salmon, at 8 °C

Figures number[a]	Medium	Vacuum	Stress	N_0	Lag	μ	N_{max}	RMSE
2a	Crushed cold-smoked salmon A	Yes	No	−0.1 [−0.9,0.7][b]	6.8 [1.9,11.7]	1.3 [0.3,2.3]	5.8 [5.1,6.4]	0.87
2a	Crushed cold-smoked salmon A	Yes	No	0.8 [−0.3,2]	3.0 [−0.5,6.6]	0.9 [0.7,1.1]	7.1 [6.6,7.5]	0.53
2a	Crushed cold-smoked salmon A	Yes	No	1.5 [0.3,3.6]	0.0 [0.0,7.5]	0.8 [0.5,1.2]	7.4 [6.8,8.0]	0.76
2b	Crushed cold-smoked salmon B	Yes	No	−0.2 [−2.2,1.9]	0.0 [0,14.3]	0.4 [0.1,0.9]	2.8 [2.3,3.3]	0.97
2b	Crushed cold-smoked salmon B	Yes	No	1.0 [0.2,1.8]	0.0 [0.0,0.0]	0.5 [0.2,0.7]	4.3 [3.9,4.6]	0.64
2b	Crushed cold-smoked salmon B	Yes	No	2.1 [1.7,2.4]	0.0 [0.0,0.0]	0.3 [0.3,0.4]	5.3 [5.1,5.5]	0.34
2c	Squared-cut cold-smoked salmon A	Yes	No	−0.3 [−0.8,0.2]	15.7 [13,19]	1.4 [0.5,2.4]	5.4 [4.0,6.7]	0.77
2c	Squared-cut cold-smoked salmon A	Yes	No	0.3 [−0.6,1.3]	0.0 [0.0,8.1]	0.7 [0.3,1.4]	5.7 [4.9,6.3]	0.81
2c	Squared-cut cold-smoked salmon A	Yes	No	2.2 [1.7,2.8]	4.9 [1.5,8.2]	0.7 [0.5,0.9]	6.8 [6.1,7.5]	0.53
2d	Crushed cold-smoked salmon A	Yes	Yes	0.1 [−0.3,0.4]	5.9 [4.4,7.5]	1.1 [0.9,1.3]	6.1 [5.8,6.4]	0.34
2d	Crushed cold-smoked salmon A	Yes	Yes	1.1 [0.7,1.5]	4.6 [3.2,5.9]	0.9 [0.8,1.0]	7.2 [6.9,7.4]	0.26
2d	Crushed cold-smoked salmon A	Yes	Yes	2.3 [1.1,3.5]	3.0 [0.0,6.9]	0.8 [0.6,1.1]	7.5 [7.0,7.9]	0.57
2e	Squared-cut cold-smoked salmon A	No	No	−0.5 [−1.4,0.4]	4.4 [0.7,8.1]	0.8 [0.6,1.0]	6.0 [5.0,7.1]	0.74
2e	Squared-cut cold-smoked salmon A	No	No	0.5 [−1.6,2.6]	2.6 [0,8.8]	0.9 [0.6,1.2]	6.7 [5.7,7.8]	0.97
2e	Squared-cut cold-smoked salmon A	No	No	2.0 [1.0,3.0]	2.7 [0,5.9]	0.8 [0.7,1.0]	8.2 [7.6,8.7]	0.48

Estimations were obtained using the logistic model with delay.
[a] Figures number indicates in which figures the curves indicated in the table are represented.
[b] Data in brackets represent 95% asymptotic confidence intervals.

that totality or part of growth parameters are equal). The risk factor α used for the F tests was 5%.

The whole analysis was performed using Mathematica software (Wolfram research, Inc.).

3. Results

Sixteen *L. monocytogenes* growth curves were performed on synthetic media, and 15 in cold-smoked salmon. The logistic model with delay provided better statistical fits to our data compared to the Baranyi model (results not shown), which was consequently abandoned. For all experimental conditions, fitted parameters for the logistic model with delay are presented in Table 1 for synthetic medium, and in Table 2 for cold-smoked salmon, together with estimated mean standard errors associated with the fitted curves. Small differences in nonlinear regression parameters between blocks, for a given experimental condition, may result from little differences in growth conditions (media pH or composition, temperature) or in the physiological state of the inoculum (stress percentage attained).

Concerning the estimated mean standard error, important differences related to both growth environment and inoculum size are observed (Tables 1 and 2), the standard errors being definitely weaker for the curves carried out in model system, than those obtained in cold-smoked salmon. Values obtained for the growth curves on solid media model ranged from 0.14 to 0.68 $\log_{10}$ CFU g^{-1}, in the case of a very low inoculum. Values obtained for the growth curves in salmon are much higher, ranging from 0.26 to 0.97 $\log_{10}$ CFU g^{-1}. Higher values of estimated mean standard errors associated with very low inoculum (<1 CFU g^{-1}) must certainly be attributed to the heterogeneity of the initial contamination (Tables 1 and 2).

For the solid model system, by performing an F test based on the likelihood ratio, we observed a significant effect of the inoculum size only for nutritionally stressed inoculum, at very low level (<1 CFU g^{-1}) with a significant increase in the lag duration (Fig. 1e and f). This effect was also observed for uninjured inoculum in the case of a growth on salmon surface in anaerobic conditions (Table 2 and Fig. 2c), but was less apparent in aerobic conditions (Fig. 2e) or crushed salmon (Fig. 2a). Moreover, this effect was no more observed for injured cells in crushed salmon (Fig. 2d). This suggests that additional effects, linked to both anaerobiosis, and the texture of the medium, had interfered with the stochastic effect of the inoculum size on the lag phase duration.

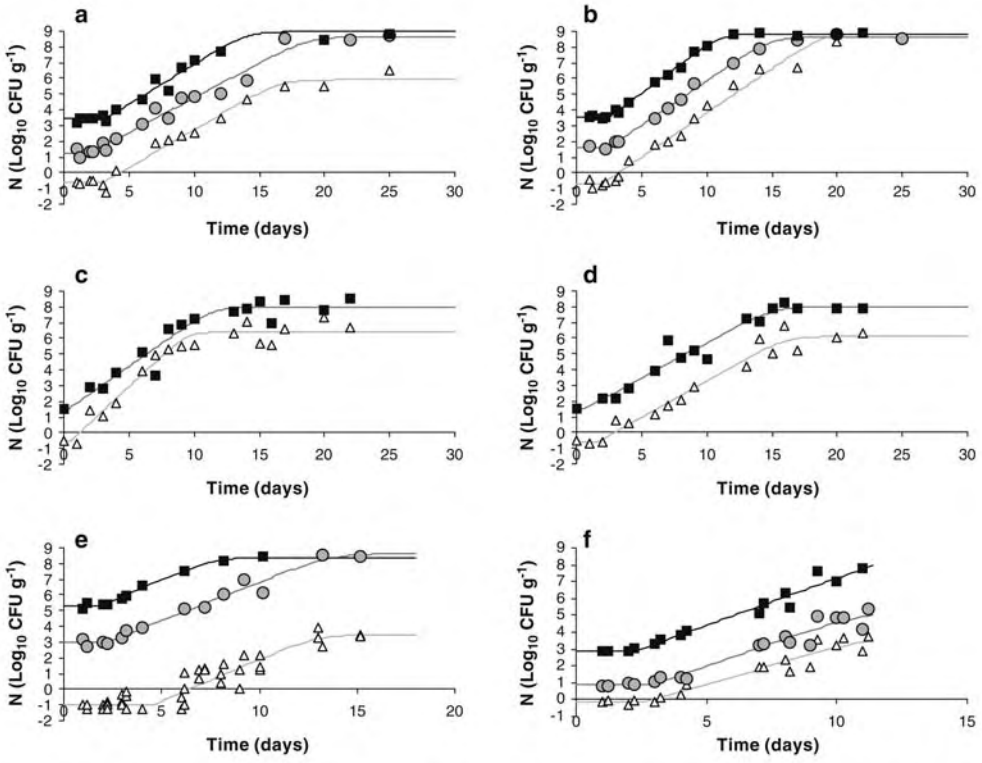

Fig. 1. Growth curves of *L. monocytogenes* in synthetic solid medium at 8 °C, under various conditions: aerobic conditions, agar medium, uninjured inoculum (a), vacuum-packaged agar medium, uninjured inoculum (b), aerobic conditions, gelatine medium, uninjured inoculum (c), vacuum-packaged gelatine medium, uninjured inoculum (d), aerobic conditions, gelatine medium, injured inoculum (e), aerobic conditions, gelatine medium, injured inoculum (f). For each condition, growth curves were performed at various initial *L. monocytogenes* population density: low inoculum (△), average inoculum (◎), high inoculum (■).In each figure, the curves are fitted using the logistic model with delay for the low inoculum (——), average inoculum (——), and high inoculum (—).

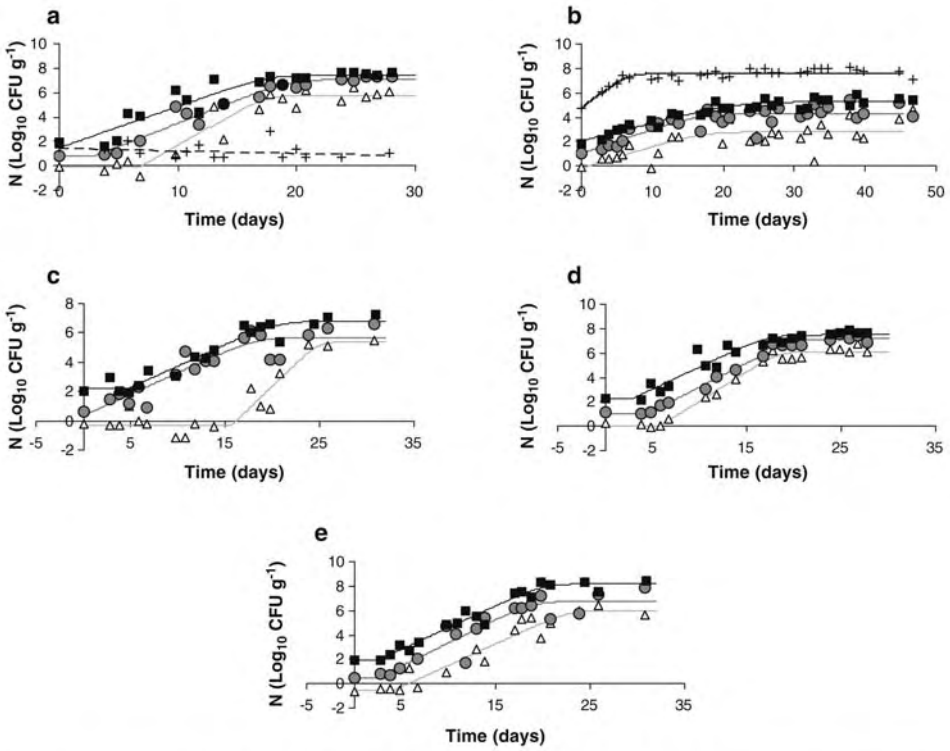

Fig. 2. Growth curves of *L. monocytogenes* and background microflora (+) in cold-smoked salmon at 8 °C, under various conditions: vacuum-packaged crushed salmon A, uninjured inoculum (a), vacuum-packaged crushed salmon B, uninjured inoculum (b), vacuum-packaged squared-cut salmon A, uninjured inoculum (c), vacuum-packaged crushed salmon A, injured inoculum (d), aerobic conditions, squared-cut salmon A, uninjured inoculum (e). For each condition, growth curves were performed at various initial *L. monocytogenes* population density: low inoculum (△), average inoculum (◎), high inoculum (■). In each figure, the curves are fitted using the logistic model with delay for the low *L. monocytogenes* inoculum (——), average *L. monocytogenes* inoculum (——), high *L. monocytogenes* inoculum (—), and background microflora (- - -).

Growth rates were quite constant for a defined medium and growth conditions, whatever the inoculum size and physiological state (Tables 1 and 2).

We observed a significant effect of the inoculum size on the maximal population attained (Fig. 3). This inoculum size effect on N_{max} was dependent on the packaging system in relation with the growth medium. For vacuum-packed agar medium, the same N_{max} was attained whatever the inoculum size (Fig. 1b), whereas, this was not the case for vacuum-packed gelatin medium (Fig. 1d), and for aerobically incubated both model systems (Fig. 1a and c). In this particular case of agar medium, vacuum-packaging seemed to accelerate the whole *L. monocytogenes* growth (Table 1, Fig. 1a and b). This unexpected result must be attributed to growth in liquid conditions. In fact, we noted the presence of a thin water film under the bag, resulting from the vacuum-package treatment. This transfer of liquid to the surface of the medium was not observed for the gelatin medium. In the case of cold-smoked salmon, we verified that vacuum packaging did not abolished the inoculum size effect on N_{max}, and slowed down growth (Fig. 2c and e).

The effect of the inoculum size on the maximal population attained was observed both for salmon A (Fig. 2a) and B (Fig. 2b), but was higher for salmon B. In fact, for salmon B, we observed an important slowing down of *L. monocytogenes* growth, whatever the level attained by the pathogen, when the background microflora reached its stationary phase (Fig. 2b). In this product, there has been an important and rapid growth of the background microflora, whereas in salmon A, the total

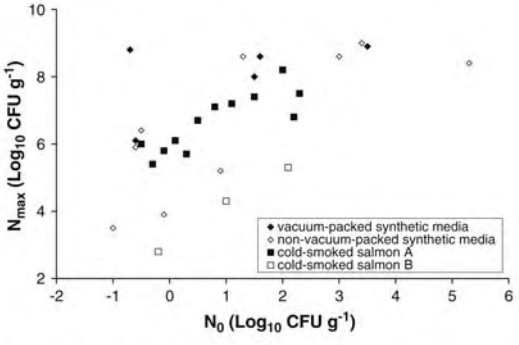

Fig. 3. Plot of N_{max} versus N_0 for each growth curve of *L. monocytogenes* on synthetic media and in cold-smoked salmon at 8 °C.

aerobic mesophilic microflora level remained extremely low during the whole experiment (Fig. 2a).

4. Discussion

This work allowed us to investigate the effect of the initial *L. monocytogenes* density on all the growth parameters of this pathogen (lag phase duration, growth rate and maximum population density attained), in structured medium. With cells severely stressed by starvation, the increase in the lag duration caused by a decrease of the inoculum size (solid model system) could be explained by an increase in the variation of individual cells lag time when cells are stressed (Baranyi and Pin, 1999; Robinson et al., 2001; Baranyi, 2002; Francois et al., 2002; Metris et al., 2003). In fact, at these very low inoculum densities, a "quorum sensing" effect seemed unlikely to occur. In our assays, the increase of the lag phase could be observed only for a very low total number of cells (<10), thanks to the use of an increased sensitivity enumeration method.

Results in cold-smoked salmon showed that additional factors may interfere with the stochastic effect of the inoculum size on the lag duration. An increase in the lag duration for very low inoculum, was observed for uninjured cells grown on the surface of cold-smoked salmon, under anaerobic conditions, but not in crushed product, or in aerobic conditions. Factors explaining the difference between growth on surface or deeply in a crushed product, and its impact on the inoculum size effect, remain to be elucidated, but could be linked to a stress caused by a lesser availability of water or nutriments. An additional stress, linked to anaerobiosis, has certainly occurred. A growth inhibition, linked to surface growth, has also been observed by Lebert et al. (2004). On the contrary, growth within the depth of crushed salmon seemed to have reduced individual cell lag variations, for injured inoculum, compared to growth on surface of synthetic medium. A better adaptation to growth in smoked salmon than in a rich synthetic medium, or a protection due to salmon components, could also have occurred in nutritionally stressed bacteria. However, this does not mean that a stochastic effect did not exist in these conditions. In fact, the high standard error associated with our results in smoked salmon could have hidden this effect, all the more if it was relatively weak. Besides, the inoculum size may have been too high in these conditions to observe a stochastic effect. All these results in cold-smoked salmon indicate the importance of taking into account food texture, when studying inoculum size effect on growth, and when performing challenge tests.

As expected, we did not find any effect of the inoculum size or physiological state on the growth rate of *L. monocytogenes*. Growth rates obtained in vacuum-packed cold-smoked salmon at 8 °C are consistent with those of different studies performed in relatively quite similar conditions (Rosso et al., 1996; Nykanen et al., 2000; Hudson and Mott, 1993; Szabo and Cahill, 1999).

The significance of the *L. monocytogenes* inoculum size on the maximal population attained in smoked fishery products has already been demonstrated in other studies (Guyer and Jemmi, 1991; Peterson et al., 1993; Pelroy et al., 1994). However, it was attributed either to interactions with other bacteria, or to the effect of food inhibitors which are more effective at lower bacterial concentrations. We found a significant effect of the inoculum size on the maximal population attained, both in synthetic media and cold-smoked salmon. In cold-smoked salmon, this effect was observed for both products (A and B), however, it was more pronounced in salmon B, which had a high level of background microflora. In this product, there has been a rapid growth of the background microflora, and we can suppose that the effect of the inoculum size on the maximal population attained has been stressed by a "Jameson effect" (Ross et al., 2000; Cornu et al., 2001). This term designates the simultaneous stop of all microflora growth in food, when the dominant bacterial population reaches its stationary phase. This phenomenon is attributed to a competition for a common nutritional resource. In fact, we observed an important slowing down of *L. monocytogenes* growth, whatever the level attained by the pathogen, when the background microflora reached its stationary phase. The more important was the *Listeria* initial contamination level, the more important was the level of the population attained at the moment of the growth inhibition. We did not observe, however, a typical "Jameson effect", since in this case, the growth stop of all bacteria would have been simultaneous and complete (Cornu et al., 2001). In the case of a bacterial growth prediction including a typical "Jameson effect", the maximal population attained by *L. monocytogenes* would have been underpredicted by 1 to 2 $\log_{10}$ CFU g^{-1}, compared to our results. One must note that the stop of *L. monocytogenes* growth could also have been caused by an acidification of the product by the background microflora.

We observed the important impact of the interactions with the food background microflora on the maximal population attained by *L. monocytogenes*, depending on the initial contamination level of the pathogen. However, our results, mainly in solid model systems, but also in cold-smoked salmon A, where the aerobic mesophilic microflora level remained very low during the whole experiment, demonstrated that an additional effect could explain this phenomenon. This effect will be called here "geometrical" effect, since it must be linked to colonial growth on solid media, and consequently to diffusion limitations of both cells, nutriments, and metabolites. Carlin et al. (1995) also found a correlation between the inoculum size and the populations reached by *L. monocytogenes*, in vegetables, which was independent of aerobic mesophilic bacteria levels. Relationships between colonial surface growth and inoculum density has been studied (Chapuis et al., 1995), but to our knowledge, the effect of the inoculum concentration on the final population attained has not been treated. Nevertheless, it has been suggested in some studies: Sanaa et al. (2004), in a quantitative risk assessment of human listeriosis linked to the consumption of soft cheeses, assumed that the colony number depends on the initial contamination, and that the maximal population attained depends on the number of colonies and of cells per colonies.

An unexpected growth stimulating effect of vacuum-packaging in the case of agar medium was observed, due to growth in liquid conditions. Even if the ability of *L. monocytogenes* to grow under vacuum storage is known, there are conflicting reports on

the potential growth of this pathogen in these conditions (Sheridan et al., 1995). These may be due to the number of factors which may affect *L. monocytogenes* growth in vacuum-packed products, one important factor being the competitive microflora (Sheridan et al., 1995). Our result in cold-smoked salmon are consistent with Peterson et al. (1993) and Pelroy et al. (1994) who showed that vacuum-packaging is an important parameter for the control of *L. monocytogenes* in smoked fishery products. However, it should be checked, for other types of products, whether vacuum-packaging could enhance the growth of the pathogen, particularly the maximal population attained, as it has been showed for the solid model system.

In relation to the quality of fit, estimated mean standard errors were much higher in cold-smoked salmon than on synthetic medium, which resulted in difficulties in the curves interpretation. Since the same artificial contamination and count procedures were applied to all growth curves, we can forward the hypothesis that this difference is explained by the heterogeneity of cold-smoked salmon, in terms of background microflora, and physico-chemical parameters. We did not verify that the use of crushed product improved the curves' quality, as suggested by Miconnet et al. (2005). Growth curves in real food were more difficult to interpret, as stressed by the quality of fit. However, the growth pattern and the inoculum size effect were different than on synthetic medium mimicking food, which suggest that taking into account chemical parameters only is insufficient to predict microbial growth in food, as stressed by Brocklehurst (2003) and Lebert et al. (2004).

In conclusion, the effect of the inoculum size on the growth of *L. monocytogenes* in structured media is dependant on a complex set of interactions. Factors which have appeared to impact on this effect include the cells physiological state, background microflora, texture of the media and the packaging system. It is important to understand how these interactions affect the growth of *Listeria* in order to predict and control its development in food. The contamination level attained by *L. monocytogenes* depends, in most cases, on the initial bacterial concentration. Consequently, when performing quantitative risk assessment studies, it is important to take into account the food contamination levels, which are generally very low, and to integrate the inoculum size effect.

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