Application of an interspecific competition model to predict the growth of *Aeromonas hydrophila* on fish surfaces during refrigerated storage

**Summary**

The growth of *Aeromonas hydrophila* and the aerobic mesophilic plate (Aeromonas hydrophila plate count) on gilthead seabream surfaces was evaluated during refrigerated storage (21 days). The related growth curves were compared with those obtained by a conventional third order predictive model obtaining a low agreement between observed and predicted data (Root Mean Squared Error = 1.77 for *Aeromonas hydrophila* and 0.64 for APC). The Lotka-Volterra interspecific competition model was used in order to calculate the degree of interaction between the two bacterial populations and respectively, the interspecific competition coefficients of APC on *Aeromonas hydrophila* and vice versa. Afterwards, the Lotka-Volterra equations were applied as tertiary predictive model, taking into account, simultaneously, the environmental fluctuations and the bacterial interspecific competition. This approach allowed to obtain a best fitting to the observed mean growth curves with a Root Mean Squared Error of 0.09 for *Aeromonas hydrophila* and 0.28 for APC. Finally, some considerations about the necessary use of competitive models in the context of the new trends in predictive microbiology were taken.

**Keywords:** *Aeromonas hydrophila*, gilthead sea bream, predictive model, bacterial interspecific competition

**Zusammenfassung**

Das Wachstum von *Aeromonas hydrophila* sowie die aerobe mesophile Gesamtkulturzahl auf der Oberfläche von Seebrassen wurden während der Kühlphase (21 Tage) ausgewertet. Die verwendeten Wachstumskurven wurden mit solchen verglichen, die durch ein konventionelles Vorhersagemodell dritter Ordnung ermittelt wurden. Letztere zeigten eine geringe Übereinstimmung zwischen vorhergesagten und beobachteten Daten (Standardabweichung = 1.77 für *Aeromonas hydrophila* und 0.64 für APC). Das Lotka-Volterra Konkurrenzmodell zwischen zwei Spezies wurde zur Berechnung des Grades der Interaktion zwischen den beiden Bakterienpopulationsen benutzt (mit den Konkurrenzkoefzienten für APC gegen *Aeromonas hydrophila* und umgekehrt (bzw. (bzw. b...)). Danach wurden die Lotka-Volterra Gleichungen als tertiäres Vorhersagemodell angewandt, wobei gleichzeitig Umweltfluctuationen und die Konkurrenz zwischen den Bakterienpopulationen berücksichtigt wurden. Dieser Ansatz erlaubte einen Fit die beobachteten mittleren Wachstumskurven mit einer Standardabweichung von 0.09 für *Aeromonas hydrophila* und 0.28 für APC. Zuletzt folgen einige Betrachtungen zur Anwendung von Konkurrenzmödellen im Kontext neuer Entwicklungen zur vorhersagenden Mikrobiologie.

**Schlüsselwörter:** *Aeromonas hydrophila*, Goldbrasse, Vorhersagemodell, bakterielle Konkurrenz
Aeromonas (A.) hydrophila (Ah) is an emerging foodborne disease agent, widely distributed in the environment. Recently, the genus Aeromonas has been classified within the family Aeromonadaceae and consists of 14 different confirmed species, one of which is A. hydrophila (Joseph and Carnahan, 2000). It is well known that the microorganism is the cause of several disease conditions in fish (fishes, amphibians), and warm-blooded (mammals and birds) animals as well as of a zoonotic disease (Daskalov, 2006). A. hydrophila is widely spread in waters, water habitats, and many food products (seafood, shellfish, raw foods of animal origin like poultry, ground meat, raw milk and raw vegetables) (Buchanan and Palumbo, 1985; Daskalov, 2006; Fricker and Tampsett, 1989; Gobat and Jemmi, 1993; Krovacak et al., 1992; Nishikawa and Kishi, 1988). With regard to foods of animal origin, according to Kumar et al. (2000) and Neyn et al. (2000), seafood products are more frequently contaminated by A. hydrophila in consequence of the wide diffusion in the aquatic environment and the ability to grow at cold temperatures. Furthermore, since A. hydrophila is an important agent of several freshwater (Kobis, 1999) and marine (Balbona et al., 1998; Zorzilla et al., 2003) reared fish diseases, its spread in the aquaculture environment could be a significant public health concern (Daskalov, 2006; Giuffrida, 2003). However, the above food safety implications are strictly related to the pathogenicity and virulence of the strain as well as to the bacterial concentration which A. hydrophila is able to reach during the storage. In the case of fish, the main A. hydrophila growth during storage, like for several other microorganisms, occurs on the skin and the gills which are considered the most important source of spoilage and pathogen bacteria (Giuffrida, 2003; Kumar et al., 2000).

The growth ability of A. hydrophila was investigated in several studies (Palumbo et al., 1985; Palumbo et al., 1991; Palumbo et al., 1992; Palumbo et al., 1996; Pin et al., 2004) which developed some predictive modelling techniques to better understand the potential behaviour of the bacteria under different kinds of food storage conditions. Nevertheless, as for other bacteria, some authors stressed the discrepancy between the growth in broth and in food supposing the natural competitive environment. Thus, fixing the growth parameters of A. hydrophila is a complex issue in the modelling of microbial evolution and it was studied by several authors. Pin and Baranyi (1998) studied the interactions of some groups of spoilage organisms that can usually be found in refrigerated meat, quantifying the inhibition exerted by a specific spoilage group (Pseudo monas spp./Serratia spp.) on each of the other groups, in the range of temperature -2 to +11 °C and pH 5.2-6.4. Coménice and Dalgaard (2004) modelled the simultaneous growth of Listeria monocytogenes and spoilage microflora in cold-smoked salmon by introducing into a simple differential equation an additional term which symbolizes the total lactic acid concentration (mechanistic inhibitory function). A further development of the study of Vereeken et al. (2003) has been recently carried out by Van Impe et al. (2005), with the introduction of the μS(t) factor which describes the influence of the phenomenon of exhaustion of a substrate (S) on the microbial evolution. Another interesting approach to the bacterial competition modelling is based on the Lotka-Volterra competition model which provides a basic equation for the population growth of two interacting species. A prototype model structure for mixed microbial populations, in food products was proposed by Dens et al. (1999) which combined the advantages of the Lotka-Volterra model for two species competition with those of the model by Baranyi and Roberts (1994) as a classical predictive growth model. Powell et al. (2004) used the aforesaid model in order to interpret some empirical results for Escherichia coli O157:H7 in ground beef and they showed that the seemingly incongruous data were consistent with the interspecific competition model. In this regard, it is important to stress that the Lotka-Volterra model can not be considered exactly like a simple "primary" predictive model (Whiting and Buchanan, 1993) since it can not be used in order to describe, directly and simultaneously, the observed microbial evolution of two competitive species as a function of time. For example, in one of the simulated scenarios of the study of Powell et al. (2004), the authors used the growth rate for Escherichia coli O157:H7, already found by Walls and Scott (1996). Afterwards, holding other factors constant, each competition model term was varied until the Theoretically Maximum Population Densities (TMD) reached the observed Maximum Population Densities (MPD). This implies, therefore, that, fixing the growth parameters of each bacterial species (such as maximum specific growth rate and physiological state of the species), the Lotka-Volterra model could be used in order to calculate the competition terms (μS and μP) of the following equations 1a and 1b) by fitting the predictive behaviours of two competitive species to the observed growth curves. However, in this case it would be necessary to use growth parameters obtained from monocolonies experimental data in order to consider only those of the interspecific bacterial competition model. The Lotka-Volterra competition model, as proposed by Dens et al. (1999) and Powell et al. (2004) is represented by the following set of differential equations:
and $N_1$ are the theoretically maximum population densities under monospecific growth conditions, $f_1$ and $f_2$ are, respectively, the interspecific competition terms of species, on species, and vice-versa; $Q_1$ and $Q_2$ represent, respectively, the physiological state of two bacterial populations.

By fixing the values of the interspecific competition terms for two bacterial populations (species, and species), it is also possible to incorporate into the Lotka-Volterra equaitons a "secondary" model which relates the maximum specific growth rates of both species to the environmental parameters ($T$, $pH$, $a_C$, $CO_2$ and $O_2$ percentage, etc.). In this way, the model will work as a "ternary" predictive model (Whiting and Buchanan, 1993) which is able to take into account, simultaneously but not only the environmentnal dynamic, like a conventional tertiary predictive model, but also the microbial community competitions.

In this work we analysed the growth of A. hydrophila on reared gilthead seabream (Sparus aurata) during refrigerated storage, comparing the observed behaviour with the predicted growth by a conventional tertiary model. Afterwards, we presented a practical application of the aforementioned theoretical approach for the evaluation of: i) the interaction intensity between A. hydrophila and the aerobic mesophilic plate count (APC), ii) the suitability of the incorporation of secondary models (polynomial equations) into the Lotka-Volterra equations in order to obtain a better fit of the observed A. hydrophila behaviour on gilthead seabream surfaces during the storage; taking also into account the fluctuations of storage temperature.

Materials and Methods

Samples and bacteriological analysis

In total 55 specimens of Sparus aurata, which resulted positive for A. hydrophila in the context of a wider research concerning the quality of marine reared fish, were analysed in order to obtain a quantitative determination of A. hydrophila and APC on skin and gills. In particular, the evaluations were carried out with regard to 63 fish with skin naturally contaminated by A. hydrophila and 32 fish with gills naturally contaminated by A. hydrophila. Samples were collected at time 0 and after 1, 2, 6, 24, 30, 36, 48, 56 hours of refrigerated storage. The storage temperature was monitored using FT-800/SYSTEM temperature data-loggers (Econorma, UK; see also Excel 2003 package, Microsoft Windows corporation). Observed and fitted growth curves were statistically analysed by using the Root Mean Squared Error (RMSE).

Conventional prediction of A. hydrophila and APC growth

The prediction of A. hydrophila and APC growth was carried out by using the conventional structure of a tertiary predictive model which combines primary and secondary models (Whiting and Buchanan, 1993). In particular, for A. hydrophila, the polynomial equation 5 (secondary model) proposed by Pin et al. (2004) and obtained from mono-cultures experimental data, was substituted into the model of Baranyi and Roberts (1994) (Eqs. 2a-b). The differential equations 2a and 2b were solved, for each growth curve, by the Runge-Kutta method and $N_{max}$ and $Q$ obtained were used as initial condition for the APC. For the APC prediction, the secondary model was constructed by acquiring several growth curves from Combase (Institute of Food Research – UK; http://wwwfood.mont.wsre.com/combase/), by using the logarithms of observed $N_{max}$ to regress to the respective temperature and pH values and a stepwise procedure was used to remove those coefficients that did not contribute significantly to the model (StatTools add-in for Microsoft Excel; Palisade Corporation, 2003). In this way the

Analysis of growth curves

For A. hydrophila 10 growth curves (5 growth curves for the skin and 5 for the gills) and as many for APC were obtained by expressing the number of colony forming units (CFU) as decimal logarithm (Log CFU/g); for the aerobic natural flora enumeration, the A. hydrophila CFU number was previously subtracted from the APC. The growth curves were analysed by the well known follow-

\[
\frac{dN}{dt} = \mu N - \frac{N^2}{N_{max}}
\]

and

\[
\frac{dQ}{dt} = \frac{\mu N}{Q_{max}} - \frac{N}{Q}
\]

were regressed to the respective temperatur and pH values and a stepwise procedure was used to remove those coefficients that did not contribute significantly to the model. For A. hydrophila growth curves, the model was fitted by fixing the values of the interspecific competition terms for two bacterial populations (species, and species), but not only the environmental dynamic, like a conventional tertiary predictive model, but also the microbial community competitions.

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coefficients $b_1 - b_2$ of the following equation 6 were obtained:

$$\ln(\frac{N_{t+1}}{N_t}) = b_1 + b_2 t + b_3 t^2$$  \hspace{1cm} (6)

where $b_1 = -2.5050$; $b_2 = -0.2267$; $b_3 = 0.0072$.

The equation 6 was substituted into the model of Baranyi and Roberts (1994) in order to construct the third order model for APC.

Both equation systems were solved numerically, by the fourth-order Runge-Kutta method, to obtain predictions for the bacterial concentration during time-dependent temperature profiles, while the other environment parameters were maintained constant ($pH=7.0$; $CO_2\% = 1.0$; $O_2\% = 20$). The initial bacterial concentrations were taken as the observed inoculum and, for the initial value of $Q_0$, the procedure of Baranyi et al. (1995) was followed for both populations. The $N_{max}$ values for $A$. hydrophila and APC (Log 7.26 CFU/g and Log 9.5 CFU/g, respectively) were obtained from the available growth curves of the ComBase online database.

The $A$. hydrophila and APC predicted growth curves were compared to the mean growth curves of the observed values of both populations and the differences were statistically analysed through the Root Mean Squared Error (RMSE).

**Lotka-Volterra competition model**

The following Lotka-Volterra competition model (Eqs. 7a-d) was used in order to analyse the interaction between $A$. hydrophila and APC and to obtain a better fitting of the theoretical results to the observed $A$. hydrophila growth on gilt-head sea bream surfaces:

$$\frac{dN_1}{dt} = \mu_1 N_1 - \frac{N_1 N_2}{K_1} - \frac{N_1 N_3}{K_2}$$  \hspace{1cm} (7a)

$$\frac{dN_2}{dt} = \mu_2 N_2 - \frac{N_2 N_1}{K_3} - \frac{N_2 N_4}{K_4}$$  \hspace{1cm} (7b)

$$\frac{dN_3}{dt} = \mu_3 N_3 - \frac{N_3 N_2}{K_5} - \frac{N_3 N_4}{K_6}$$  \hspace{1cm} (7c)

$$\frac{dN_4}{dt} = \mu_4 N_4 - \frac{N_4 N_2}{K_7} - \frac{N_4 N_3}{K_8}$$  \hspace{1cm} (7d)

where $N_1$ and $N_2$ are, respectively, the population densities of $A$. hydrophila and APC at time $t$, $\mu_1$ and $\mu_2$ are the maximum specific growth rates of both populations, $N_{max}$ and $N_{max}$ are the theoretically maximum population densities under monoculture growth conditions, $f_{max}$ and $f_{max}$ are, respectively, the inter-specific competition terms of APC on $A$. hydrophila and vice-versa, $Q_{Ah}$ and $Q_{PA}$ represent, respectively, the physiological state of two bacterial populations.

Analogously to the above conventional third order models, equations 5 and 6 were substituted, respectively, into equations 7a-b and 7c-d allowing the instantaneous modification of growth rate during time-dependent temperature profiles; also in this case the other environment parameters were maintained constant ($pH=7.0$; $CO_2\% = 1.0$; $O_2\% = 20$). $N_{max}$ values of both populations were the same as those used for the construction of the conventional third order model.

The system was solved numerically by the fourth-order Runge-Kutta method and, at the same time, the Solver of Microsoft Excel (Office 2003 package, Microsoft Windows corporation) was used in order to fit the predicted $A$. hydrophila and APC behaviours to the mean growth curves of the observed values, by modifying both $B$-terms ($B_{APC}$ and $B_{PA}$, respectively in equations 7a and 7c).

Also in this case, the $A$. hydrophila and APC predicted growth curves were compared to the mean growth curves of the observed values and the differences were statistically analysed through the Root Mean Squared Error (RMSE).

Note that this approach allows to consider only once the effect of interspecific bacterial competition ($B$-term) since, as explained in the above section, the other parameters derive from monoculture experimental data.

FIGURE 1: Observed behaviour of $A$. hydrophila (■) and Aerobic Plate Count (▲) during refrigerated storage at fluctuating temperature (---) of gilt-head seabream. Red and blue straight lines indicate, respectively, the predicted growth of $A$. hydrophila (---) and Aerobic Plate Count (---) using the conventional third order model approach.

**Results and Discussion**

The observed mean growth curves for $A$. hydrophila and APC as well as the population behaviours predicted by the conventional tertiary models are showed in Figure 1; in the same figure is also reported the mean recorded temperature during the refrigerated storage. The mean growth parameters estimated for the $A$. hydrophila growth curves on fish surfaces show a very slow increase with a
FIGURE 2: Observed behaviour of A. hydrophila lines indicate, respectively, the predicted growth of A. hydrophila bic Plate Count (---) during refrigerated storage at fluctuating temperature (---) of gilthead seabream. Red and blue straight lines indicate, respectively, the predicted growth of A. hydrophila and Aerobic Plate Count (---) using the Lotka-Volterra interspecific competition model.

Figure 2 shows the predictions obtained by the incorporation of equations 5 and 6 into the Lotka-Volterra model as well as the mean of the observed data and the mean temperature records. In this case the model allowed to take into account, simultaneously, the environmental influences (temperature fluctuations) on bacterial growth and the interspecific competitions (β terms Tab. 1) which are concentration dependent. As Figure 2 (RMSE values) shows, this approach produced a better fitting of predicted to observed data, especially concerning the reaching of Maximum Population Density. It is important to stress that the proposed model allows to express the behaviour of two competitive bacterial populations as the result of two opposed forces: the former is represented by the growth rate of each population which derives from monoculture experimental data; the latter is the interspecific competition term (β) of each population on the other one which is related to the bacterial growth.

Finally, we can conclude that the proposed interspecific competition model represents a good solution in order to consider, at the same time, the complexity of the food substrate during storage (fluctuating environmental condition) and the interspecific bacterial interactions. On the contrary, conventional predictive tertiary models which reproduce the mono-specific bacterial growth in food where the natural flora has a high increase during the storage, could produce incongruous data. This matter would be considered by the new trends in predictive microbiology since, as we showed, the bacterial interspecific competitions can significantly affect the population behaviour.

It is evident that the limit of the present work is represented by considering APC as a single bacterial population since it is well known that it includes several kinds of aerobic bacterial species. However, the aim of this work was to characterise the behaviour of A. hydrophila on gilthead seabream surfaces, taking into account the competitive effect of all other bacterial species. On the contrary, the eventual characterisation of the competitive effect of only one spoilage aerobic species (e.g. Pseudomonas spp.) on A. hydrophila growth could produce a result with a minor applicative meaning. In this regard, a further development of the present study could consider the modelling of several competitive species (more than two) as theoretically proposed by Fiasconaro et al. (2006).

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