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Predictive models as means to quantify the interactions of spoilage organisms

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Abstract

The purpose of this paper is to quantify the interactions of some groups of spoilage organisms that can be usually found in refrigerated meat stored in air, such as: Enterobacteriaceae, *Pseudomonas*, *Acinetobacter*, *Psychrobacter*, *Shewanella*, *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *Brochothrix* and *Kurthia* spp. The growth of these organisms was studied in the range of temperature 2–11°C and pH 5.2–6.4, which is characteristic of refrigerated meat. The main growth parameters (maximum specific growth rate and lag time) were modelled by multivariate quadratic polynomials of temperature and pH. The interactions of the organisms were analyzed by comparing their growth models obtained in isolation with those obtained in mixture. The difference between the models was quantified by statistical *F*-values which were used to measure how much the growth of an organism or group of organisms was affected by others and which of them dominated their joint growth.

Keywords: Predictive microbiology; Spoilage; Interactions; *Pseudomonas*; *F*-test

1. Introduction

Predictive food microbiology has been focusing on modelling the microbial responses to the food environment, in the interest of food safety and of avoiding spoilage. Predictive models have been regularly published to describe the growth of a strain or a mixture of strains as a function of the environmental conditions characteristic of food.

Because of the variety and number of spoilage organisms, spoilage models are less straightforward to develop than pathogen models and their application is much more limited. Dalgaard (1995) suggested that predictive models for spoilage should be developed only after knowing the micro-organisms responsible for the reactions important in the process of spoilage and the range of environmental conditions under which these organisms cause spoilage. McMeekin and Ross (1996) emphasised that spoilage models, based on the responses of the dominant organism, are valid only in a specific range of

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conditions. Out of this range, different bacteria or metabolites may be responsible for the spoilage, making the model no longer valid.

The process of spoilage in aerobically stored chilled meat is well characterised and this food system was chosen for our work. *Pseudomonas*, *Acinetobacter* and *Psychrobacter* spp. are the dominant bacteria causing spoilage, when their concentration reaches $\sim 10^7/\mathrm{cm}^2$, or /g, as a consequence of their fast growth and their metabolism. *Brochothrix thermosphacta*, cold-tolerant Enterobacteriaceaea and lactic acid bacteria also occur in aerobically-stored meat but generally show slower growth and/or cause less offensive or later spoilage signs (Gill and Newton, 1977; Dainty and Mackey, 1992; Lambropoulou et al., 1996).

The growth conditions that can be found in chilled meat are: high water activity (~ 0.99); pH values of ~ 5.8 though they can go from ~ 5.4 to ~ 7.0, and refrigeration temperatures (Egan and Roberts, 1987). The process of chilled meat spoilage can be identified as the growth of the aerobic gram-negative bacteria or simply of *Pseudomonas* spp.

Most foods are complex systems with heterogeneous populations. A mechanistic approach describing the interactions of different strains in food would require a complex mathematical model with many variables. Here, we do not make an attempt to describe the dynamic coexistence of organisms in meat but we take a simpler approach. We analyse the differences between the isolated and joint growth of four groups of spoilage organisms, that can be usually found in refrigerated meat stored aerobically. The results enables us to quantify how much the growth of a group is affected by the other groups and to what extent one group dominates the spoilage flora. We define a subpopulation of a mixture of organisms dominant if its specific growth rate is higher than that of the other subpopulations. A dominant subpopulation does not always outnumber the others because the actual number of cells depends also on the lag phase and the inoculum size. However, these latter parameters depend on the initial conditions (the lag depends on the initial physiological state) and, in fact, the maximum specific growth rate is the only intrinsic parameter of bacterial growth. If one of the subpopulations has higher maximum specific growth rate than the others, then it outnumbers the others provided approximately equal inoculum size and initial physiological state at the start.

After comparing the growth models, a generic spoilage model is created for the specific growth rate, by using the data of the groups found as dominant. In accord with the above note on the concept of dominance, the generic model can help to predict the time to spoilage assuming that the dominant organisms are present in sufficient number at all.

2. Materials and methods

2.1. Bacterial strains

Thirty-two strains of *Pseudomonas*, *Acinetobacter*, *Psychrobacter*, *Shewanella*, Enterobacteriaceae, *Carnobacterium*, *Lactobacillus*, *Leuconostoc*, *B. thermosphacta* and *Kurthia* spp., were used in this work. They were divided into four groups as follows.

Group P: *Pseudomonas putida* NCFB 754 (spoiled milk), *Pseudomonas fragi* NCFB 2902 (beef), *Pseudomonas lundensis* NCFB 2908 (minced beef), *Shewanella putrefaciens* NCFB 756 (tinned butter), *Acinetobacter* sp. NCIMB 11168 (lamb carcass meat), *Acinetobacter* sp. NCIMB 11169 (lamb carcass meat), *Psychrobacter inmobilis* NCIMB 11372 (pork sausage), *Psychrobacter immobilis* NCIMB 11650 (meat).

Group E: *Enterobacter agglomerants* NCFB 2071 (minced meat), *Enterobacter agglomerants* NCFB 2073 (pasteurised milk), *Klebsiella oxytoca* NCFB 2678 (Cheddar cheese), *Klebsiella pneumoniae* NCFB 1010 (unknown), *Escherichia coli* NCFB 555 (raw milk), *Proteus morganii* NCTC 235 (unknown).

Group L: *Lactobacillus* sp. NCFB 2812 (vacuum packed pork), *Lactobacillus* sp. NCFB 2813 (vacuum packed beef), *Lactobacillus* sp. NCFB 2814 (vacuum packed bacon), *Lactobacillus* sp. NCFB 2815 (vacuum packed lamb), *Leuconostoc carnosum* NCFB 2775 (vacuum packed meat), *Leuconostoc gelidum* NCFB 2776 (vacuum packed meat), *Leuconostoc gelidum* NCFB 2800 (vacuum packed beef), *Carnobacterium divergens* NCFB 2856 (vacuum packed beef), *Carnobacterium divergens* NCFB 2857 (vacuum packed lamb), *Carnobacterium piscicola* NCFB 2853 (vacuum packed beef), *Carnobacterium piscicola* NCFB 2854 (vacuum packed lamb).

Group B: *Brochothrix thermosphacta* NCFB 1676 (fresh pork sausage), *Brochothrix thermosphacta* NCFB 2830 (lamb), *Brochothrix thermosphacta* NCFB 2849 (vacuum packed pork), *Kurthia gibsonii* NCIMB 10499 (pork sausage), *Kurthia zopfii* NCIMB 10494 (hamburgers), *Kurthia zopfii* NCIMB 10496 (frozen mince pork), *Kurthia zopfii* NCIMB 10498 (pork sausage), *Kurthia gibsonii* NCIMB 10495 (fat trimmings), *Kurthia gibsonii* NCIMB 10497 (rendered lard).

2.2. Media

Tryptone Soya Broth (TSB, Oxoid/Unipath CM129) was made in 3000-ml volumes, adjusted to the target pH values using 5 M HCl, dispensed in 250-ml volumes and autoclaved at 121°C for 15 min.

The bacterial counts were obtained on: Tryptone Soya Agar for non-selective counts (TSA, Oxoid CM 131); Cetrimide Fusidin Cephaloridine agar for *Pseudomonas* spp. (CFC, Oxoid CM559, SR 103); MacConkey agar for Enterobacteriaceae (Oxoid/Unipath CM115); MRS agar for lactic acid bacteria (MRSA, Oxoid CM 361); Streptomycin Thallous Acetate Actidione agar for *B. thermosphacta* (STAA, Oxoid CM 881, SR151).

2.3. Inoculum preparation

The cultures of each strain were activated by transferring loop inocula grown in 10 ml of TSB at 25°C at three successive 24-h intervals immediately prior to the experiments. Equal volumes of the cultures, prediluted approximately to 10^5 cfu/ml, were combined to give cocktails of the groups P, E, L, and B. Equal volumes of this cocktail were used to get a total mixture (T) containing all the strains together.

2.4. Experimental procedure

TSB was maintained overnight at the intended storage temperature. After measuring the pH, the broth was inoculated with the appropriate cocktail giving a final concentration of $\sim 10^3$ cfu/ml. Immediately after inoculation, the broth was aseptically dispensed in 10-ml volumes, using a peristaltic pump (Accuramatic Mk 5; Jencons Ltd., Leighton Buzzard, UK), into sterile 1-oz universal bottles with plastic screw caps. The initial inoculum level was determined from one of the bottles and the remaining bottles were incubated at the desired temperature (± 0.5°C) in static conditions. During the storage, the samples were plated onto either selective or non-selective agars, according to Table 1. Plates were incubated for 24–48 h at 25°C, using anaerobic jars (Oxoid/Unipath, HP11) with Gas Generating Kits (Oxoid/Unipath BR038B) when required.

Growth responses were studied under 12 conditions, combining four temperatures (2, 5, 8 and 11°C) and three pH values (5.2, 5.8 and 6.4), as shown in Table 1.

The ability of a selective agar to recover a certain strain was checked by growing the strain at 25°C, for

Table 1
Organisation of the 'type A', 'type B' models and the total model. Each model is based on growth curves generated at the 12 combinations of four temperatures (2, 5, 8, 11°C) and three pH values (5.2, 5.8 and 6.4)

	Model code	Group of organisms inoculated	Growth media in broth	Plating media	Plates incubation
Type A	M(P)	P	TSB	TSA	Aerobic
	M(E)	E	TSB	TSA	Aerobic
	M(L)	L	TSB	MRSA	Anaerobic
	M(B)	B	TSB	TSA	Aerobic
Type B	M(T,CFC)	Total (all the four groups inoculated together)	TSB	CFC	Aerobic
	M(T,MacC)		TSB	MacConkey[a]	Aerobic
	M(T,MRSA)		TSB	MRSA	Anaerobic
	M(T,STAA)		TSB	STAA	Aerobic
Total model	M(T,TSA)	Total	TSB	TSA	Aerobic

[a]Only red colonies were counted to select group E.

24 h, then plating it on the selective agar (Table 2, first five columns).

Each strain was incubated at the four temperatures for 30 days to establish whether they were able to grow at those temperatures at all (Table 2, last column).

2.5. Growth models

The generated bacterial growth curves were fitted by the model of Baranyi and Roberts (1994) (see Fig. 1a and b). The main growth parameters, estimated at each combination of temperature and pH, were:

- maximum specific growth rate (μ)
- lag phase (λ).

When a mixture of organisms grow together, the specific growth rate is not constant, even if all the subpopulations are in the exponential phase. The instantaneous specific growth rate of the i-th subpopulation, at the time t, is

$$\mu_i(t) = \frac{\frac{d}{dt}x_i(t)}{x_i(t)}$$

where $x_i(t)$ is the respective bacterial concentration. Let the overall concentration be denoted by

$$x(t) = x_1(t) + x_2(t) + \ldots$$

Then, as it can be derived mathematically, the instantaneous specific growth rate of the whole population, at the time t is:

$$\mu(t) = \mu_1(t)\frac{x_1(t)}{x(t)} + \mu_2(t)\frac{x_2(t)}{x(t)} + \ldots$$

i.e. the weighted average of the individual specific rates, where the weights are the actual proportions of the individual sub-concentrations in the whole population. Therefore, assuming that the fastest growing subpopulation does not have longer lag and smaller starting number than the others, the dominance in rate means numerical dominance very soon and the specific rate of the whole population becomes practically indistinguishable from the fastest specific growth rate. This justifies the use of the model of Baranyi and Roberts (1994), to fit growth curves of mixed cultures, since that model is based on the assumption that the specific growth rate is practically constant for a phase.

To correct the heterogeneity of the variance, the natural logarithm (ln) of μ and λ was modelled as a function of temperature and pH. This model was a standard multivariate second order polynomial as described by McClure et al. (1993). About the use of the variance-damping log-transformation, see Alber and Schaffner (1992).

Each of the nine growth models created was denoted by a code referring to the name of the modelled group and possibly to the used selective media (Table 1). The model codes do not include any reference to the modelled growth parameter and, unless we explicitly state in another way, the term 'model' refers to ln μ.

Two types of predictive models characterising the four groups and a total model were generated for comparisons.

Type A: a group inoculated in isolation. The four models of 'type A' were denoted by M(P), M(E), M(L) and M(B), respectively.
Type B: all the above groups inoculated together in a total mixture (T) and plated on selective agar. The four models of 'type B' were denoted by M(T,CFC), M(T,MacC), M(T,MRSA) and M(T-,STAA), respectively.
Total model: all the above groups inoculated together in a total mixture (T) and plated on non-selective, ordinary TSA. The model obtained this way is denoted by M(T,TSA).

After comparing the growth models, also a generic spoilage model, M(G), was created for the specific growth rate, by using the data of the groups found as dominant.

2.6. Measuring microbial interactions

(a) Differences between the growth rates of the groups in isolation were studied by comparing the models M(P), M(E), M(L), and M(B) with each other.

(b) To study how the growth of a particular group was affected by the total mixture, its polynomial model for ln μ ('type A') was compared with the model obtained on its respective selective medium from the total mixture ('type B'):

M(P) vs. M(T,CFC)
M(E) vs. M(T,MacC)
M(L) vs. M(T,MRSA)
M(B) vs. M(T,STAA)

(c) To identify the dominant group in the total mixture, the models for both ln μ and λ obtained on selective media ('type B') were compared with those obtained on the non-selective TSA (Total model):

M(T, TSA) vs. M(T,CFC), M(T,MacC), M(T,MRSA), M(T,STAA)

2.7. F test

An F test was used to decide whether the difference between two models was significant. The procedure of the test can be summarised as follows.

Suppose that a series of n observations, $y_1 \ldots y_n$, (in our case, growth rates or lag times under various environmental factors) are fitted by the model M_1 with p_1 parameters. Similarly, let a series of m observations, $y_{n+1} \ldots y_{n+m}$, be fitted by the model M_2 with p_2 parameters, and let M_2 have similar structure (such as polynomial) as M_1. Furthermore, let the merged $n+m$ observations, $y_1 \ldots y_{n+m}$, be fitted by a unified model, M_u, with p_u parameters, and with the same model structure as M_1 and M_2. In other words, we have

1. a separated model (M_s) of $p_s = p_1 + p_2$ parameters containing two segments, M_1 and M_2, laid over the observations $y_1 \ldots y_n$ and $y_{n+1} \ldots y_{n+m}$, respectively;
2. a unified model (M_u) of p_u parameters laid over the unified observation points, $y_1 \ldots y_{n+m}$.

Under general conditions imposed on the variance of the data, the question, whether the difference between the models M_1 and M_2 is significant, can be tested by the F value (Box and Draper, 1987)

$$F = \frac{\dfrac{\sum_{i=1}^{n+m} (\hat{\hat{y}}_i - \hat{y}_i)^2}{p_s - p_u}}{\dfrac{\sum_{i=1}^{n+m} (y_i - \hat{y}_i)^2}{n + m - p_u}}$$

where $\hat{y}_i$ is the fitted value for the i-th observation when the separated model, M_s, is used for fitting and $\hat{\hat{y}}_i$ is the fitted value when the unified model, M_u, is applied.

When the probability belonging to an F value was less than 0.05 ($P < 0.05$), the difference between the two models was considered significant.

3. Results

3.1. Recovery of the organisms on selective media

The recovery of the different organisms on the used selective media is shown in Table 2.

CFC was used to recover the group P from the total mixture. The *Acinetobacter* and *Psychrobacter* strains of the group P did not grow on CFC but did grow on MacConkey agar giving red colonies. Three strains from the group E were able to grow on CFC agar. Among the bacteria of the group P, the *Pseudomonas* and *Shewanella* strains grew on CFC, while the *Acinetobacter* and *Psychrobacter* strains did not. The group P grown alone and the total mixture plated on CFC showed similar results. This indicates that the *Pseudomonas* and *Shewanella* strains were primarily responsible for the growth within this group when inoculated separately. This was supported by the fact that one *Acinetobacter* strain grew neither at 2°C nor at 5°C and one *Psychrobacter* strain did not grow at 2°C (Table 2). Hence the failure to recover the *Acinetobacter* and *Psychrobacter* strains on CFC did not affect the comparison between M(P) and M(T,CFC).

Moreover, the *Acinetobacter* and *Psychrobacter* strains gave red colonies on MacConkey agar. As it will be shown, the growth of those bacteria giving red colonies on MacConkey agar was thoroughly inhibited in the total mixture. Because of this strong inhibition, the mentioned four strains of the group E did not have any effect on the counts obtained on CFC agar.

For these reasons, *Pseudomonas* and *Shewanella* strains were considered to be the organisms responsible for the growth of the group P when inoculated alone. Besides, CFC agar could be used to measure the growth of the group P in the total mixture.

MacConkey agar was used to recover the group E from the total mixture, counting red colonies only. *Pseudomonas* and *Shewanella* strains (from the

Table 2
Recovery of the different strains of the four groups on selective media

Microorganisms	Logcounts of culture, grown at 25°C, for 24 h, on:					Lowest growth temperature studied (°C)
	TSA	CFC	McConkey	MRSA	STAA	
Group P:	9.6	9.5	9.5 (R+Y)	ND	ND	
Pseudomonas putida NCFB 754	10	10	9.7(Y)	ND	ND	2
Pseudomonas fragi NCFB 2902	10	9.9	9.9(Y)	ND	ND	2
Pseudomonas lundensis NCFB 2908	9.8	9.9	9.9(Y)	ND	ND	2
Shewanella putrefaciens NCFB 756	9.6	9.0	ND	ND	ND	2
Acinetobacter sp. NCIMB 11168	9.8	ND	9.6(R)	ND	ND	8
Acinetobacter sp. NCIMB 11169	9.8	ND	9.8(R)	ND	ND	2
Psychrobacter inmobilis NCIMB 11372	9.7	ND	9.7(R)	ND	ND	2
Psychrobacter inmobilis NCIMB 11650	9.6	ND	9.6(R)	ND	ND	5
Group E:	10	9.0	9.9(R)	5.25	ND	
Enterobacter agglomerants NCFB 2071	9.7	ND	9.5(R)	6.28	ND	2
Enterobacter agglomerants NCFB 2073	9.4	ND	9.5(R)	ND	ND	2
Klebsiella oxytoca NCFB 2678	10	9.9	10(R)	ND	ND	2
Klebsiella pneumoniae NCFB 1010	10	10	10(R)	ND	ND	2
Escherichia coli NCFB 555	10	ND	9.9(R)	ND	ND	8
Proteus morganii NCTC 235	9.4	9.7	9.4(Y)	ND	ND	2
Group L:	9.1	ND	ND	9.3	ND	
Carnobacterium divergens NCFB 2856	8.8	ND	ND	9.2	ND	2
Carnobacterium divergens NCFB 2857	9.3	ND	ND	9.4	ND	2
Carnobacterium piscicola NCFB 2853	9.0	ND	ND	9.2	ND	2
Carnobacterium piscicola NCFB 2854	9.3	ND	ND	9.5	ND	2
Lactobacillus sp. NCFB 2812	9.2	ND	ND	9.3	ND	2
Lactobacillus sp. NCFB 2813	9.6	ND	ND	9.6	ND	2
Lactobacillus sp. NCFB 2814	9.1	ND	ND	9.3	ND	2
Lactobacillus sp. NCFB 2815	9.3	ND	ND	9.4	ND	5
Leuconostoc carnosum NCFB 2775	9.2	ND	ND	9.5	ND	2
Leuconostoc gelidum NCFB 2776	9.7	ND	ND	9.4	ND	2
Leuconostoc gelidum NCFB 2800	9.3	ND	ND	9.4	ND	2
Group B:	9.2	ND	ND	ND	9.2	
Kurthia gibsonii NCIMB 10499	8.4	ND	ND	ND	ND	5
Kurthia zopfii NCIMB 10494	8.3	ND	ND	ND	ND	2
Kurthia zopfii NCIMB 10496	9.0	ND	ND	ND	ND	2
Kurthia zopfii NCIMB 10498	8.7	ND	ND	ND	ND	2
Kurthia gibsonii NCIMB 10495	8.7	ND	ND	ND	ND	2
Kurthia gibsonii NCIMB 10497	9.2	ND	ND	ND	ND	2
Brochothrix thermosphacta NCFB 1676	8.8	ND	ND	ND	8.7	2
Brochothrix thermosphacta NCFB 2830	8.3	ND	ND	ND	8.0	2
Brochothrix thermosphacta NCFB 2849	8.7	ND	ND	ND	9.0	2

ND, growth not detected; (Y), yellow colonies; (R), red colonies.

group P) gave yellow colonies on MacConkey agar. This made it difficult and inaccurate to count the red colonies. In spite of all these problems, the results on MacConkey agar let us measure the growth of the group E in the total mixture.

MRSA worked quite well as a selective medium for the group L.

On STAA, *Kurthia* spp. could not grow unlike *B. thermosphacta*. When the group B was grown in isolation, *Kurthias* spp. did not grow. This was concluded from the result that we observed the same growth curves when incubating the plates of the group B under aerobic (both *Brochothrix* and *Kurthia* spp. were able to grow) and anaerobic (only

Brochothrix spp. could grow) conditions. Hence, the growth of the group B was attributed exclusively to *B. thermosphacta*, and STAA was used to measure the growth of the group B in the total mixture.

3.2. Isolated growth of the groups (data for models of 'type A')

The maximum specific growth rates (μ) and the lag times (λ) of the groups P, E, L and B, when grown in isolation, were derived by the curve fitting procedure of Baranyi and Roberts (1994). The logcount curves of the four groups, grown at 2°C and pH 5.8, are shown in Fig. 1a, for an example. The fitted growth parameters, for every studied combination of temperature and pH, are shown in Table 3.

Fig. 1a suggests that the group L is slower than the other three. The group P has the fastest growth but its rate is just slightly higher than that of the group B which, on the other hand, has the longest lag. The question to be answered is whether these observations are true for other temperature and pH values.

Fig. 2a represents, in 3-D, the maximum specific growth rates of Table 3 as a function of temperature and pH. It shows that the observation made about the group L in Fig. 1a, namely that it grows more slowly than the others, is true only at low temperatures. In the main, however, Table 3 and Fig. 2a confirm what Fig. 1a demonstrated about the difference between the growth rates of the four groups.

When comparing the 'type A' models with each other (specific growth rate models obtained in isolation), the *F*-test results showed no significant differences between group P and E ($P=0.33$). The specific growth rate of group B was also similar. The difference between groups B and E was not significant ($P=0.37$). Between groups P and B, it was significant at 5% but not at 1% ($P=0.021$). The model of group L, however, was significantly different ($P<0.05$) from each of the other three models.

3.3. Growth of the groups in the total mixture (data for models of 'type B')

Fig. 1b shows the fitted growth curves produced by the total mixture when plated on four different agars each selective for the four groups. This figure also shows a fifth fitted curve which was obtained on the non-selective TSA, from the total mixture.

Fig. 1b suggests that the growth of the group P (selected, from the total mixture, by CFC agar) is not

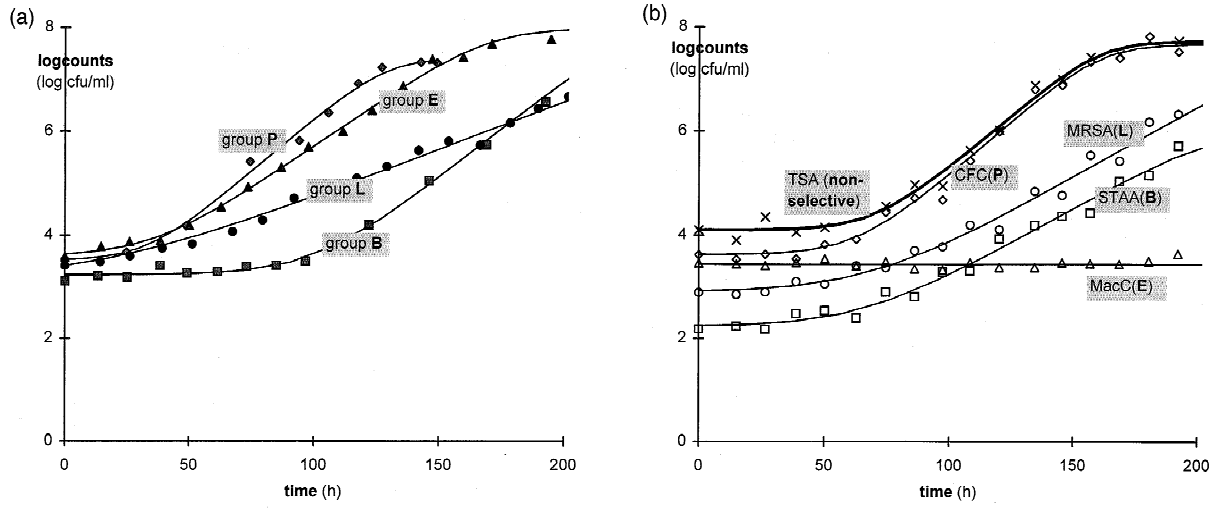

Fig. 1. (a) Fitted logcounts obtained when each of the four groups was grown in isolation, at 2°C, pH 5.8. Curves like these give the basis for the models of 'type A'. ♦, group P; ▲, group E; ●, group L; ■, group B. (b) Fitted logcounts obtained when the total mixture of the four groups was grown at 2°C, pH 5.8 and plated on the four selective agars (thin lines) and on TSA (thick line). Curves like these give the basis for the models of 'type B' (thin lines) and for the total model (thick line). The name of the group, for which an agar is selective, is in parenthesis in the figure. The legend is parallel to (a), according to the group-agar relation: ◇, CFC; △, MacConkey; ○, MRSA; □, STAA; ×, total mixture plated on TSA.

Table 3
Maximum specific growth rates (1/h) and lag times (h) of the groups inoculated in isolation. These growth parameters provided the input data for the models of 'type A'

Temp	pH	Group P				Group E				Group L				Group B			
(°C)		μ	Rel. err.	λ	Rel. err.	μ	Rel. err.	λ	Rel. err.	μ	Rel. err.	λ	Rel. err.	μ	Rel. err.	λ	Rel. err.
2	6.4	0.090	7.9%	30	22%	0.071	4.2%	37	12.0%	0.044	3.6%	31	25%	0.11	6.8%	67	6.3%
2	5.8	0.084	5.7%	29	18%	0.072	3.0%	36	8.0%	0.042	2.8%	30	22%	0.084	7.1%	98	6.2%
2	5.2	0.076	6.5%	48	13%	0.063	3.7%	44	8.4%	0.034	2.7%	26	33%	0.068	3.6%	89	4.2%
5	6.4	0.12	12.0%	32	21%	0.081	4.3%	24	19.0%	0.066	3.9%	21	28%	0.11	3.5%	44	6.5%
5	5.8	0.11	6.3%	29	15%	0.077	3.9%	20	22.0%	0.059	2.7%	20	21%	0.086	4.9%	64	7.2%
5	5.2	0.11	8.3%	46	11%	0.077	4.2%	41	8.9%	0.049	3.7%	23	33%	0.079	2.6%	110	2.3%
8	6.4	0.20	7.0%	16	13.0%	0.20	9.6%	24	10.0%	0.14	3.3%	10	21%	0.18	2.9%	18	8.5%
8	5.8	0.19	7.0%	16	14.0%	0.17	12.0%	24	13.0%	0.14	2.4%	10	14%	0.16	3.0%	28	5.7%
8	5.2	0.15	3.3%	18	7.9%	0.17	7.6%	28	7.7%	0.12	2.1%	11	13%	0.14	4.9%	43	6.2%
11	6.4	0.28	7.9%	7.0	26%	0.26	9.7%	12	12.0%	0.29	6.0%	2.2	95%	0.26	7.3%	8.7	29%
11	5.8	0.27	5.5%	4.3	36%	0.26	6.5%	11	10.0%	0.29	4.2%	2.9	50%	0.24	3.6%	10	13%
11	5.2	0.22	8.3%	6.8	41%	0.22	5.6%	11	14.0%	0.27	3.1%	2.8	43%	0.31	13.0%	27	10%

μ, maximum specific growth rate (1/h).

λ, lag time (h).

Rel. err., standard error of the fitted parameter expressed as percentage of the parameter.

different from that of the total mixture plated on TSA. The other groups grew slower than the group P, and the group E did not grow in the experimental time at all.

The growth parameter estimates, as produced by the curve fitting described above, can be seen in Table 4, for every studied combination of temperature and pH. That table, with Fig. 2b, demonstrates that the observations made on the specific growth rates in Fig. 1b are typical for the whole region of environmental factors analyzed here.

While the group P was the dominant in the mixed

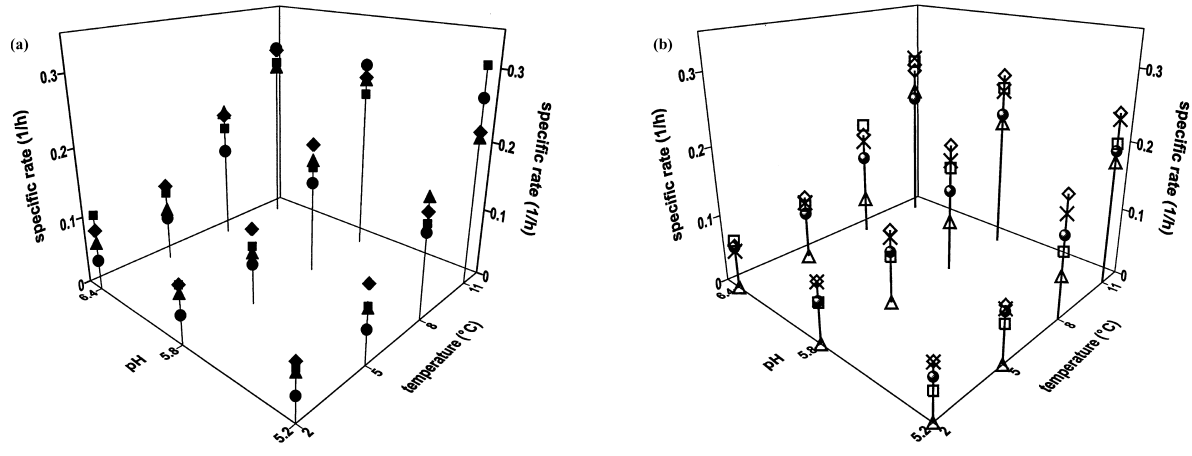

Fig. 2. (a) Maximum specific growth rates (μ, see Table 3) of the four groups grown in isolation. ♦, group P; ▲, group E; ●, group L; ■, group B. (b) Maximum specific growth rates (μ, see Table 4) of the total mixture plated on the four selective agars and on TSA. ◇, CFC; △, MacConkey; ○, MRSA; □, STAA; ×, total mixture plated on TSA.

Table 4
Maximum specific growth rates (1/h) and lag times (h) of the total mixture plated on selected agars (first four sections) and on TSA (last section). These growth parameters provided the input data for the models of type B and the total model, respectively

Temp	pH	CFC(P)				MacC(E)				MRSA(L)				STAA(B)				TSA(non-selective)			
(°C)		μ	Rel. err.	λ	Rel. err.	μ	Rel. err.	λ	Rel. err.	μ	Rel. err.	λ	Rel. err.	μ	Rel. err.	λ	Rel. err.	μ	Rel. err.	λ	Rel. err.
2	6.4	0.065	7.1%	32	31%					0.062	4.3%	59	10.0%	0.072	4.7%	50	12%	0.057	5.5%	23	44%
2	5.8	0.087	7.8%	57	10%					0.060	3.6%	66	7.5%	0.058	9.4%	62	16%	0.086	10.0%	66	10%
2	5.2	0.075	15.0%	64	20%					0.056	2.6%	73	5.1%	0.040	15.0%	82	16%	0.074	11.0%	74	13%
5	6.4	0.10	6.0%	24	17%					0.071	4.5%	32	16.0%	0.085	3.9%	19	28%	0.089	4.2%	21	15%
5	5.8	0.11	8.4%	35	13%					0.076	5.6%	45	12.0%	0.069	7.3%	23	33%	0.097	6.5%	34	11%
5	5.2	0.080	9.1%	29	30%					0.072	3.8%	43	10.0%	0.055	19.0%	43	30%	0.075	8.4%	31	28%
8	6.4	0.17	5.1%	13	20%	0.10	11.0%	12	26%	0.13	2.8%	5.4	59.0%	0.18	4.0%	12	16%	0.16	5.1%	12	22%
8	5.8	0.19	6.8%	20	11%	0.076	4.8%	12	28%	0.12	2.4%	7.9	28.0%	0.16	5.1%	12	22%	0.17	5.5%	17	14%
8	5.2	0.17	5.7%	22	9.0%	0.063	7.9%	8.5	63%	0.12	2.0%	8.9	20.0%	0.10	5.6%	8.3	46%	0.15	6.5%	18	15%
11	6.4	0.25	7.8%	8.5	27%	0.21	4.3%	9.3	15%	0.20	2.2%	4.7	27%	0.26	4.1%	5.7	28%	0.27	10.0%	9.8	21%
11	5.8	0.27	8.6%	9.4	21%	0.20	5.3%	7.0	30%	0.21	2.2%	4.2	30%	0.25	10.0%	8.6	34%	0.25	8.2%	8.5	25%
11	5.2	0.25	6.3%	10	15%	0.18	4.4%	8.1	20%	0.20	2.0%	4.1	29%	0.21	12.0%	8.9	34%	0.24	9.4%	9.3	25%

population for all of the conditions, the group E did not grow at 2°C and 5°C.

3.4. Comparing models of 'type A', 'type B' and the total model

To measure how other organisms affected the growth of a particular group, its maximum specific growth rate was compared with those obtained on selective agar from the total mixture. This comparison was carried out on the specific growth rate values associated to the filled and hollow symbols in Fig. 2a and b, respectively.

To measure which group was the dominant in the total mixture, the maximum specific growth rates obtained on the respective selective media are to be compared with those obtained on TSA from the total mixture (see the difference between the hollow symbols and the 'cross' symbol in Fig. 2b). Lag phases were analyzed in the same way.

The comparison is even more efficient when carried out on fitted response surfaces. Those were generated from the data represented by:

— the hollow symbols of Fig. 2a
(gaining 'type A' models: M(P), M(E), M(L) and M(B));
— the filled symbols of Fig. 2b
(gaining 'type B' models: M(T,CFC), M(T,MacC), M(T,MRSA) and M(T,STAA));
— the 'cross' symbols of Fig. 2b
(gaining the total model: M(T,TSA)).

The fitted coefficients of the above quadratic models for ln μ and ln λ, as well as the respective standard errors of fitting are tabulated in Table 5.

To establish whether the difference between two response surfaces was significant, the *F* test was applied. By that, for each group, we could obtain quantified answers to the following questions.

1. Did the group grow in a significantly different way in the total mixture than in isolation? (Comparing its 'type A' model with the respective 'type B' model.)
2. Can its growth be identified with the total growth? (Comparing its 'type B' model with the total model.)

The *F*-value can be considered as a certain 'distance' between two models, therefore the higher the *F*-value is, the more reason we have to believe that two compared models are different. High *F*-values indicate negative answers to the above two questions, which means, in our case: (i) inhibitory effect of the other organisms on the group in

Table 5
Coefficients of the quadratic surfaces fitted to the natural logarithm of the specific growth rates (ln μ) and the lag times (ln λ)

	Model code	Growth parameter	Coefficients						S.E. of fitting
			Const	Temp	pH	Temp.pH	$Temp^2$	pH^2	
Type A	M(P)	Ln μ	−6.911	0.04097	1.369	0.01182	0.001505	−0.1109	0.066
		Ln λ	31.39	−0.1110	−9.231	0.05013	−0.02932	0.7517	0.122
	M(E)	Ln μ	−4.777	0.04475	0.5848	0.008529	0.004685	−0.04605	0.209
		Ln λ	21.46	−0.1508	−5.830	0.03131	−0.01194	0.4709	0.211
	M(L)	Ln μ	−9.409	0.2006	1.780	−0.01923	0.01035	−0.1295	0.099
		Ln λ	−1.469	0.3519	1.428	−0.03801	−0.03041	−0.1066	0.074
	M(B)	Ln μ	0.1956	0.2776	−1.476	−0.05605	0.01390	0.1750	0.109
		Ln λ	10.84	0.3834	−1.974	−0.07063	−0.01438	0.1533	0.207
Type B	M(T,CFC)	Ln μ	−16.90	0.0633	4.920	0.005542	0.003724	−0.4270	0.133
		Ln λ	−8.734	−0.3371	5.047	0.03325	−0.002973	−0.4827	0.121
	M(T,MacC)	Ln μ	−5.833	0.3708	0.02260				0.149
		Ln λ	2.097	−0.07262	0.1365				0.140
	M(T,MRSA)	Ln μ	−6.184	0.06183	1.056	−0.004472	0.008024	−0.08525	0.062
		Ln λ	0.1225	−0.4628	2.021	0.02395	−0.0004022	−0.2034	0.396
	M(T,STAA)	Ln μ	−14.08	0.2029	3.252	−0.02444	0.008459	−0.2324	0.134
		Ln λ	4.847	−0.6598	0.5918	0.03870	0.01485	−0.09711	0.282
Total model	M(T,TSA)	Ln μ	−14.14	−0.1173	4.112	0.02870	0.007475	−0.3694	0.136
		Ln λ	−7.275	−0.7663	5.060	0.09737	0.001154	−0.5249	0.179
	M(G)[a]	Ln μ	−12.65	−0.004356	3.467	0.01535	0.004234	−0.3024	0.126

[a]Generic spoilage model for ln μ based on the growth rates of the dominant organisms.

question; (ii) that the considered group is not among the dominant ones in the total growth.

3.4.1. Results comparing 'type A' and 'type B' models

Fig. 3 and Fig. 4 compare the models M(P) and M(E) with M(T,CFC) and M(T,MacC), respectively. Note that, in the case of M(T,MacC), the response model is a linear surface because growth was detected at the highest two temperatures only. From Fig. 3 and Fig. 4, it can be concluded that the growth of the group P was not affected by the other groups, but the group E grew significantly slower in the total mixture than in isolation.

The distances between the models were expressed in terms of the *F* test results, as can be seen in Fig. 5.

Fig. 5 demonstrates that the models of the groups E, L and B, when grown in isolation (denoted by M(E), M(L) and M(B), respectively), showed significantly faster growth than those obtained on MacConkey, MRSA and STAA from the total mixture (denoted by M(T,MacC), M(T,MRSA), and M(T,STAA), respectively). This confirms that there is no significant difference between the growth models of the group P, whether grown in isolation or in the total mixture, and that the biggest discrepancy was found between the two models of the group E,

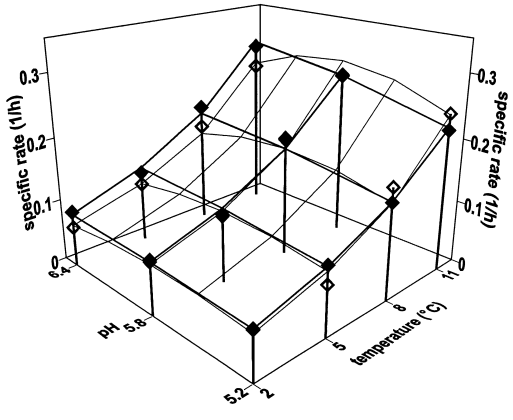

Fig. 3. Comparing the growth of group P in isolation with that in the total mixture. (Models M(P) and M(T,CFC)). Legend as in Fig. 1a and Fig. 1b. Thick surface: M(P). Thin surface: M(T,CFC).

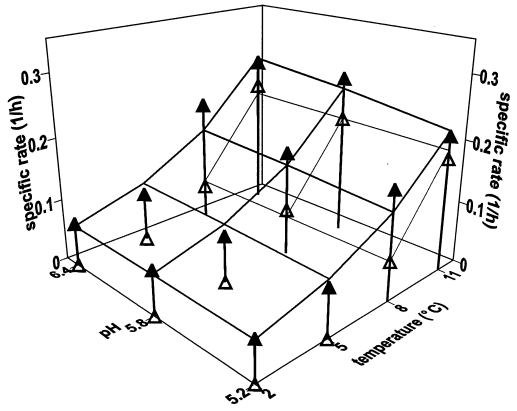

Fig. 4. Comparing the growth of group E in isolation with that in the total mixture. (Models M(E) and M(T,MacC)). Legend as in Fig. 1a and Fig. 1b. Thick surface: M(E). Thin surface: M(T,MacC).

indicating that the other groups severely affected the growth of group E.

3.4.2. Results comparing 'type B' models and the total model

The dominant group responsible for the growth in the total mixture was identified by determining not only the group or groups with the fastest growth rate but also with the shortest lag phase. Therefore, the *F* test was carried out on the estimated growth rates (Fig. 6) as well as on the lag times (Fig. 7).

Fig. 6 demonstrates that there was no significant difference between the model of the group P in the total mixture, M(T,CFC), and the total model, M(T,TSA). The growth of the rest of the groups was significantly slower than that of the total mixture. Hence, the group P was the dominant in the total mixture, and its model was the closest to M(T,TSA) while the model of the group E, M(T,MacC), was the furthest away.

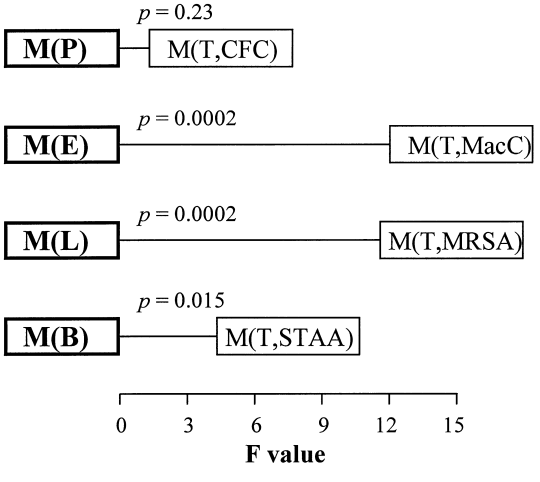

Fig. 5. Representing the difference between 'type A' (thick boxes) and 'type B' (thin boxes) models of the maximum specific growth rate by *F*-values. The respective *P*-values show the probability of that high *F*-value occurring by chance when the compared models are equal.

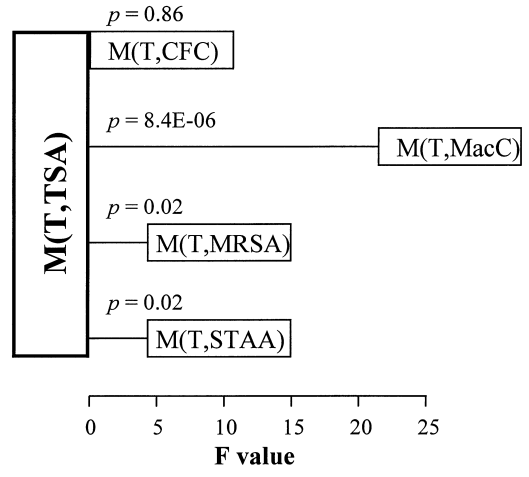

Fig. 6. Representing the difference between the total (thick box) and 'type B' (thin boxes) models of the maximum specific growth rate by *F*-values.

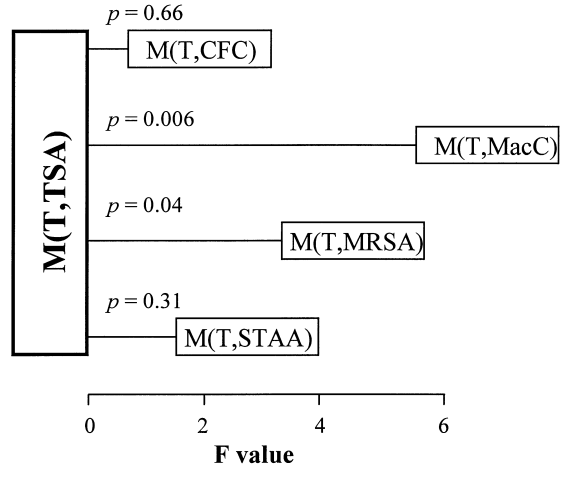

Fig. 7. Representing the difference between the total (thick box) and 'type B' (thin boxes) models of the lag by *F*-values.

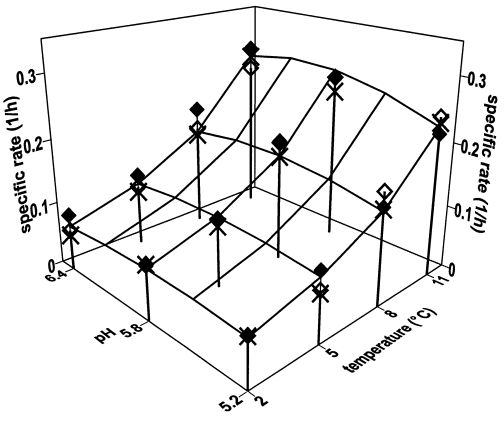

Fig. 8. The generic model, M(G), fitted on the specific growth rates of (i) the group P grown alone ('type A' model); (ii) the group P grown in the total mixture and selected by CFC agar ('type B' model); (iii) the total mixture plated on TSA (total model). Legend as in Fig. 1a and Fig. 1b.

The obtained *F*-values for the lag, comparing 'type B' models with the total model, are shown in Fig. 7. As the figure shows, the relation of the lag models are similar to that of the growth models.

3.5. Creating a generic spoilage model for the maximum specific growth rate

The generic spoilage model of the mixed population, M(G), based on the dominant group of the total mixture, was created by fitting those three datasets of ln μ, for which the *F* test showed no significant differences (Fig. 8):

- maximum specific growth rates of the group P, when grown in isolation;
- maximum specific growth rates of the total mixture plated on CFC;
- maximum specific growth rates of the total mixture plated on TSA.

The obtained coefficients and their standard errors for this generic model are shown in Table 5.

4. Discussion

Simple quantification and easy visualisation of the differences between predictive models is not new in the literature. The coefficients of predictive models, describing the growth rate of some *Aspergillus* spp. as a function of a_w, was used by Baranyi et al. (1997) as a measure of the relatedness of microbial species. Dengremont and Membré (1995), applied the percentage similarity between the specific growth rates of different *Staphylococcus aureus* strains to represent the distances between them. In our case, *F* test values were employed for a similar purpose.

The *F* values quantifying the discrepancy between 'type A' and 'type B' models (Fig. 5) can establish an order between the groups in terms of level of inhibition. This order from the least to the most affected group can be summarised as group P< group B<group L<group E.

In the mixed population (T), the closest model to M(T,TSA) is M(T,CFC), while M(T,MacC) is the most distant. Using the *F* values of Fig. 6 and Fig. 7 in a similar manner as above, a rank can be established between the bacterial groups according to their contribution to the growth of the mixed population: group P>group B≥group L>group E.

Therefore, the dominant organism or organisms, in the mixed population, could be one or more of the strains of the group P, which were able to grow on CFC (*P. putida*, *P. fragi*, *P. lundensis* and/or *S. putrefaciens*). The rest of the spoilage groups were significantly inhibited in the mixed population.

Dainty and Mackey (1992) raised the question whether the rapid growth of *Pseudomonas* spp. might be a sufficient reason for their dominance in some chilled food products. In our work, no significant differences were detected in the growth rates of the groups P, E and B when grown in isolation. Since the only difference in the growth conditions of the total mixture was the presence of the other groups, microbial interactions must be the cause of the reduction of the growth rates. We showed this reduction for all the organisms except for the group P, which was able to grow at the same rate in the total mixture as in isolation.

Gill and Newton (1977) suggested that pseudomonads may have a greater affinity for oxygen than facultatively anaerobic bacteria, which would result in the reduction of the growth rates of the latter group. However, this effect can be noticed at high concentration of pseudomonads only.

The ability of *Pseudomonas* species to transform glucose rapidly to a form not readily utilised by the competing organisms has been considered as a competitive advantage (Whiting et al., 1976a,b). Indeed, the sequestering of glucose as gluconate might be one of the reasons for the success of *Pseudomonas* spp. (Nychas et al., 1988). However, as Dainty and Mackey (1992) wrote, the inhibitory effect of the glucose transformation would be apparent only when glucose becomes limiting which is not the case in our broth system.

Nychas et al. (1988) also mentioned that, beside the fast growth rates, the tolerance to acidity might be another reason for the success of *Pseudomonas* spp. However, Enterobacteriaceae, *Moraxella*, *Aeromonas*, *Acinetobacter* or *Flavobacterium* spp. are as tolerant to acidity as *Pseudomonas* spp. within the range pH 5.5–6.4 (Gill, 1986).

Gram (1993) suggested that one of the causes for the bacterial selection in the microflora of some food products is the influence of microbial interactions such as competition or antagonism. She found that most strains of *Shewanella putrefaciens* were strongly inhibited by *Pseudomonas* strains isolated from spoiled and fresh fish. The inhibitory effect was associated with siderophore-mediated competition for iron. However, that inhibition was not observed in liquid media, only in agar assays, using cultures of pseudomonads pregrown to maximum cell numbers.

Our work cannot clarify the reasons for the dominance of *Pseudomonas* spp. We can conclude only that the reason for this dominance is not exclusively their fast growth. Neither can it be that some substrates become limiting for the competing organisms since they were dominant, from the beginning of the growth of the total mixture, in a medium rich in nutrients, at relatively low bacterial concentrations.

It is worth discussing the propagation of errors through the modelling procedure. In Table 3 and Table 4, the growth parameters were obtained through a non-linear curve-fitting procedure which also estimated the standard error of those parameters. These errors are expressed in the table as percentages of the estimated parameters themselves (relative errors). As can be seen, the relative errors for the maximum specific growth rates are fairly constant and are between 5% and 15%, dominantly below 10%. The constant relative error justifies the use of the logarithm as a link function (McGullagh and Nelder, 1983) when the maximum specific growth rates were modelled as a function of temperature and pH.

In Table 5, the standard errors of those fitted quadratic response surface models can be seen. Those errors are around 0.1 which indicates that the specific rates are estimated by about $\exp(0.1) = 1.1$ factor accuracy. This means that the relative standard error of that estimation is about 10%, a result which is consistent with the overall picture of the relative errors of the specific rates given in Table 3 and Table 4. In other words, the applied (in fact, purely empirical) quadratic polynomial model was ‘good enough’ in the studied region of the environmental factors because it did not introduce significant model error.

The reason why we considered only the maximum specific growth rate in this comparative study, and not the lag time, is the doubtful reproducibility of the latter parameter (Shida et al., 1977; Buchanan and Klawitter, 1991; Hudson, 1993).

Bacterial interactions and competition is extensively studied in literature. An inherent problem of these studies is that the quantity and quality of the available data do not match with the number of variables and the complexity of the mathematics required by mechanistic models. Panikov (1996) used four differential equations to model the steady-state growth and transient dynamics of a mixed population of pseudomonads and enterobacteria. To simulate the competition of 2–5 species for a common limiting substrate and the growth on several substrates, a model containing up to 22 differential equations was needed. The more accurately we want to describe a real system, the higher the number of necessary variables becomes, and the more complex the mathematical model will be.

In this paper, our goal was not a mechanistic modelling of competition. Our approach was to quantify the concepts of ‘dominance’ and ‘influence’ of spoilage bacteria on each other. This aim was compatible with the data that we could measure with traditional microbiological techniques. However, further studies are necessary not only to identify the kind of interactions that can be established in a mixed culture, but also, to find a less laborious and

more accurate way of studying the behaviour of different organisms in heterogeneous populations.

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