Growth and metabolic features of lactic acid bacteria in media with hydrolysed fish viscera. An approach to bio-silage of fishing by-products.

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Headline: Lactic acid bacteria productions in hydrolysed fish viscera media.

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ABSTRACT

The reparameterization of two classic growth models (the logistic and Gompertz equations), and the dynamic modification of the integrated form of the first of these, was applied to the description of the kinetics and metabolic behaviour of six strains of lactic acid bacteria in four media: three of these from visceral waste from fishing products, and one commercial medium. The descriptions obtained –always consistent models and statistically significant parameters– provided a wide range of reliable numerical values on notable characteristics of microbial growth and bioproductions, which allowed the assessment of the individual systems by direct comparison, and also the suggesting of the potentially most suitable groups (of species of fish and of bacteria) for bio-silage processes.

Keywords: lactic and acetic acids, logistic equation, marine peptones, nitrogen source, fish viscera, lactic acid bacteria silage, fish bio-silage.

INTRODUCTION

The commercial media (ATP, MRS, TGE) usual for the production of lactic acid bacteria (LAB) and their metabolites, are characterised by a composition which is especially rich in salts and, particularly, in proteins, both due to their concentration and to their diversity of origin (tryptone, bactopeptone, yeast extract, meat extract, frequently several of these at the same time). From an industrial point of view, the use of these media, which are very costly, reduces the profitability of the large-scale culture

of LAB, whether it is for producing probiotic biomass, bacteriocins, lactic and acetic acids or starters for biological silage processes.

Peptones –widely used in microbiological media and defined as water-soluble, non heat-coagulable protein hydrolysates which contain a mixture of peptides, proteoses and free amino acids (Green et al., 1977)– come mainly from casein, soya, gelatine and meat. Over the last years, however, fish peptones have aroused a growing interest for a number of reasons, among which are their good results in the same applications as traditional peptones, their suitability for inclusion in *kosher* and *muslim* products (Aspmo et al., 2005; Horn et al., 2005), and the absence of the reservations which the risk of spongiform encephalopathies arouses in meat peptones (Aspmo et al., 2005a; Horn et al., 2005; Wells et al., 1987), and the possible content of genetically modified organism-derived materials in vegetable peptones.

In previous studies (Vázquez et al., 2004a; Vázquez et al., 2006a) it was shown that trout, swordfish, tuna or cephalopod waste allow the simple, rapid preparation of protein hydrolysates or autohydrolysates suitable for the formulation of culture media for LAB, which equal or surpass the biomass and bacteriocin productions obtained in high-priced commercial media. The same waste is also a suitable substrate for biological silage with lactic acid bacteria (Lindgren and Refai, 1984; Lassen, 1995; Ahamed and Mahendrakar, 1996; Yoon and Lee, 1997; Uchida et al., 2004), the result of which is a material applicable to the formulation of high-quality fish flours, such as those used as a protein base in the feed for aquaculture purposes.

However, the approaches to the silage of fish applied until now are the result of eminently empirical viewpoints, based on traditional techniques such as the preparation of fish sauces, the ripening of anchovies or the silage of vegetable matter such as grass (Viet and Bello, 1989; Dong et al., 1993; Faid et al., 1994; Martínez-Valdivieso et al., 1994; Weinberg et al., 2002; Weinberg and Ashbell, 2003). The typification of these techniques is difficult because the microbial populations present are mixed and their metabolic capabilities depend not only on the species involved but also on the characteristics of the medium (as is the case with the homo- and heterofermentative metabolism of LAB), and on interspecific interactions. Finally, the process must guarantee the displacement and destruction of the undesirable microbiota which may be present, by means of the conditions imposed on the system by the development of the inocula or starters added (Ahamed and Mahendrakar, 1996; Dapkevicius et al., 2000; Viet and Bello, 1989).

The possibility of having low-cost media, suitable for the culture of a microbiota with a demand for peptidic sources as high as that of LAB (Aspmo et al., 2005a; Aspmo et al., 2005b; Duffossé et al., 2001; Gao et al., 2006; Horn et al., 2005; Vázquez et al., 2004a; Vázquez et al., 2006a) would, in the first place, facilitate the preparation of the relatively large-scale inocula required by the silage of fish waste. On the other hand, the aforementioned complexity of the silage process makes those media a simpler model of the system, but whose microbial dynamics allow the definition of the factors which establish its most useful goal: the repeatable obtaining of microbiologically safe products of a nutritionally suitable composition, reducing the probability of undesired deviations and improving the desired characteristics of their final state.

In this context, the aims of this study include: 1) the preparation of a media as an alternative to the well-known commercial MRS, by means of the replacement of its protein content by other low-cost protein sources obtained from fish waste, 2) the establishment and systematic application of various mathematical models, as a necessary conceptual tool for the carrying out of relevant, reliable comparisons between cultures, based on parameters with clear biological meaning and quantified statistical significance, 3) the kinetic assays of six LAB (Table 1) on these waste media quantified growths and lactic and acetic acid concentrations (bioproductions with implications in the bio-silage process).

MATERIALS AND METHODS

1: Preparation of marine peptones from fish viscera

Raw materials used were viscera from swordfish (*Xiphias gladius*), shark (*Isirus oxyrhinchus*) and thornback ray (*Raja clavata*), sampled immediately after industrial processing and maintained (15 days at most) at –20ºC until use. Visceral masses (stomach and intestine) were ground with ~100% (v/w) of distilled water, stabilised by steam flow (101ºC/1 hour) and treated in a centrifuge decanter at 6,000 rpm/15 minutes to obtain the corresponding sediments and supernatants. Supernatants (marine peptones) were typified according to the levels of total nitrogen, protein and total sugars, and then stored at –20ºC until the time of its use in the formulation of culture media. The basic composition of peptones is shown in Table 2.

2: Microbiological methods

The micro-organisms used, together with their origins and abbreviated keys, are shown in Table 1. Stock cultures were stored at –75ºC in MRS medium (Hispanlab) with 25% glycerol (Cabo et al., 2001a). Inocula (1% vol/vol) consisted of cellular suspensions from 24-hour and 12-hour (Lc HD1) aged cultures on MRS medium, adjusted to an OD (λ=700 nm) of 0.900.

The compositions of the media are summarized in Table 3. Each marine peptone was used at a level that replaced the Lowry protein concentration present in the commercial MRS medium. In all cases, the initial pH was adjusted to 7.0 and the solutions were sterilised at 121ºC for 15 min. Cultures were carried out in triplicate, using 300 mL Erlenmeyer flasks with 200 mL of medium, at 30ºC with 200 rpm orbital shaking.

3: Analytical methods

At pre-established times, each culture was divided into two aliquots. The first aliquot was centrifuged at 5,000 rpm for 15 minutes, and the sediment washed twice and resuspended in distilled water at an appropriate dilution to measure the optical density at 700 nm. The dry weight can then be estimated from a previous calibration curve. The corresponding supernatant was used for the determination of proteins, lactic and acetic acids and reducing sugars. The second aliquot was used for the quantification of viable cells by means of a plate count technique on MP and MRS agar media. Serial tenfold dilutions were prepared in peptone-buffered solutions, and 0.1 mL samples were plated in quadruplicate, incubated at 30ºC for 48-72 h, and manually counted. Results were expressed as colony-forming units per mL (cfu/mL).

Additional analyses (in duplicate) were: *Total nitrogen*: method of Havilah et al. (1977), applied to digests obtained by the classic Kjeldahl procedure. *Proteins*: method of Lowry et al. (1951). *Total sugars*: phenol-sulphuric reaction (Dubois et al., 1956), according to the method of Strickland and Parsons (1968), with glucose as a standard. *Reducing sugars*: 3,5-dinitrosalicylic reaction (Bernfeld, 1951). *Lactic and acetic acid*: HPLC, after membrane filtration (0.22 µm Millex-GV, Millipore, USA) of samples, using an ION-300 column (Transgenomic, USA) with 6 mM sulphuric acid as a mobile phase (flow=0.4 mL/min) at 65ºC and a refractive-index detector.

4: Mathematical models and numerical methods

4.1: Growth and production models

Two equations which are widely used to describe microbial growth are –see notations in Table 4– the logistic model (1) and the Gompertz model (2) (Arino et al., 2006; Lejuene et al., 1998; Dalgaard and Koutsoumanis, 2001; Murado et al., 2002; Vázquez et al., 2003; Vázquez et al., 2004b; Vázquez et al., 2004c; Wachenheim et al., 2002):

$$X = \frac{K}{1+\exp\left(c-\mu_{mx}\cdot t\right)} \;\; ; \;\; c = \ln\left(\frac{K}{X_0}-1\right) \qquad (1)$$

$$X = K\cdot\exp\left[-\exp\left(b-a\cdot t\right)\right] \qquad (2)$$

However, forms (1) and (2), in spite of being habitual in this respect, present drawbacks when, in addition to the simple formal description, statistically significant comparisons between parameters of practical interest are attempted. In fact, *K* is in both cases the

asymptote (maximum biomass), and, in the logistic model, μ_{mx} represents the maximum specific rate, a datum of less importance than the maximum rate, and more sensitive to experimental error. Certainly, important data such as maximum rate (v_{mx}), or the lag period (λ_x), may be obtained according to the fitting parameters, but this complicates the calculation of the confidence intervals (Vázquez et al., 2006b; Paz et al., 2006). Thus, the best solution is to reparameterize form (1) in order to make explicit the parameters of interest, whose confidence intervals are in this case easily obtained by means of computer applications such as *Statistica* or *MatLab*:

$$X = \frac{K}{1+\exp\left[2+\frac{4\cdot v_{mx}}{K}\cdot\left(\lambda_x - t\right)\right]} \qquad (3)$$

Naturally, the same equation is applicable to other productions with similar temporal profiles of interest in this context (Vázquez and Murado, 2008 in press). Thus, the biomass measured in terms of cfu (*N*), lactic acid (*L*) and acetic acid (*A*), whose parameters are shown in table 5, affected by the subscripts corresponding to the variable considered (see also notations in Table 4).

With regard to the form (2), Zwietering et al. (1990) reparameterized this model with the same criteria as discussed in the previously work (Vázquez and Murado, 2008 in press) for the case of the logistic equation. Applying the same procedures for the calculation of the slope of the straight tangent to the function (v_{mx}) at its inflection point (t_i):

$$\frac{d^2X}{dt^2} = K\cdot a^2\cdot\exp\left[-\exp\left(b-a\cdot t\right)\right]\cdot\exp\left(b-a\cdot t\right)\cdot\left[\exp\left(b-a\cdot t\right)-1\right]$$

$$\frac{d^2X}{dt^2}=0 \quad \xrightarrow{t=t_i} \quad t_i=\frac{b}{a} \tag{4}$$

$$v_{mx}=\left(\frac{dX}{dt}\right)_{t_i}=K\cdot a\cdot\exp\left[-\exp\left(b-a\cdot\frac{b}{a}\right)\right]\cdot\exp\left(b-a\cdot\frac{b}{a}\right)=\frac{K\cdot a}{\exp(1)} \tag{5}$$

The calculation of the lag period implies the same operations described for the logistic model (Vázquez and Murado, 2008 in press):

$$X\left(t_i\right)=X\Big|_{t=t_i}=K\cdot\exp\left[-\exp\left(b-a\cdot\frac{b}{a}\right)\right]=\frac{K}{\exp(1)}=\frac{K}{e}$$

$$R=X\left(t_i\right)+f'\left(t_i\right)\cdot\left(t-t_i\right)=X\left(t_i\right)+\left(\frac{dX}{dt}\right)_{t_i}\cdot\left(t-t_i\right)=\frac{K}{e}+v_{mx}\cdot\left(t-t_i\right)$$

$$\frac{K}{e}+v_{mx}\cdot\left(\lambda_x-t_i\right)=0 \quad\Rightarrow\quad \lambda_x=\frac{v_{mx}\cdot t_i-\frac{K}{e}}{v_{mx}}=\frac{\frac{K\cdot a}{e}\cdot\frac{b}{a}-\frac{K}{e}}{\frac{K\cdot a}{e}}=\frac{b-1}{a} \tag{6}$$

Finally, the definitive expression is obtained by inserting the values of v_{mx} and λ_x given by (5) and (6) into (2):

$$X=K\cdot\exp\left\{-\exp\left[\frac{e\cdot v_{mx}}{K}\cdot\left(\lambda_x-t\right)+1\right]\right\} \tag{7}$$

It should be pointed out that with growths assessed in terms of biomass (dry weight) the Gompertz equation (7) led to lower numerical fitting than the logistic equation (3), while being very effective with growths expressed as logarithms of the normalised values of the cfu, *i.e.*, $\ln(N/N_0)$. In such a case, however, the parameter which translates the slope at the inflection point (v_{mx}) has the dimensions of the inverse of a time, making

it necessary to reinterpret the same as a maximum specific growth rate, *i.e.*, as μ_{mx} in the logistic equation (1). Similarly, the parameter *K* loses its meaning as maximum number of cfu, and becomes an adimensional parameter *B*. In this way, if the dependent variable is $\ln(N/N_0)$, the reparameterised Gompertz model should be written:

$$\ln\frac{N}{N_0} = B\cdot\exp\left\{-\exp\left[\frac{\mu_{mn}\cdot(\lambda_n - t)\cdot e}{B}+1\right]\right\} \qquad (8)$$

and the maximum value of *N* (that is, N_m) should be calculated as:

$$\lim_{t\to\infty}\ln\left(\frac{N_m}{N_0}\right) = B\cdot\exp\left[-\exp\left(-\infty+1\right)\right] = B \quad \Rightarrow \quad N_m = N_0\cdot\exp\left(B\right) \qquad (9)$$

4.2: Luedeking and Piret model

In order to typify the metabolic nature of the different bioproductions of the biomass, we turned to the combination of the above models with that of Luedeking and Piret (1959a; 1959b), which links the growth rate with the rate of production of a particular metabolite. Thus:

$$r_p = \frac{dP}{dt} = \alpha\cdot\frac{dX}{dt} + \beta\cdot X = \alpha\cdot r_x + \beta\cdot X \qquad (10)$$

which permits the classification of the metabolites as primary (rate of formation dependent only on the rate of biomass production: $\alpha\neq 0$; $\beta=0$), secondary (rate of formation dependent only on the biomass present: $\alpha=0$; $\beta\neq 0$), and mixed (rate of

formation dependent on the rate of biomass production and on the biomass present at the same time: $\alpha \neq 0$ y $\beta \neq 0$).

An additional typification involved the calculation of the yields and specific productivity between productions and consumptions (Vázquez and Murado, 2008, in press):

$$Y_{P/AR} = \frac{\Delta P}{\Delta AR} = \frac{P_f - P_i}{AR_i - AR_f} \quad (11)$$

$$Y_{P/Pr} = \frac{\Delta P}{\Delta Pr} = \frac{P_f - P_i}{Pr_i - Pr_f} \quad (12)$$

Finally, it should be pointed out that MRS medium contains acetic acid, and that both the inocula and the fish peptones necessarily contain low, but detectable levels of organic acids. For this reason, for the adjustment of the experimental results to the models proposed, those initial levels were subtracted from those established analytically at each sampling time, in order to obtain strictly the net productions, and to avoid possible biases in the parametric values derived from a non-null intercept.

4.3: Numerical and statistical methods

Fitting procedures and parametric estimations calculated from the results were carried out by minimisation of the sum of quadratic differences between observed and model-predicted values, using the non linear least-squares (quasi-Newton) method provided by the macro 'Solver' of the Microsoft Excel XP spreadsheet. Statistica 6.0 (StatSoft, Inc. 2001) and Simfit v.5.6.7 (kindly provided by Dr. W.G. Barsley, Manchester University, UK and Dr. F.J. Burguillo, Salamanca University, Spain) programs were used to

determine the statistical significance of the parametric estimations, the consistence of the proposed models and to perform the cluster analysis.

RESULTS AND DISCUSSION

1: Production of biomass, organic acids, and nutrient consumption in complete media.

The experimental results, of which one representative example is shown in figure 1, highlighted a great variety of responses depending on the microbial strain and on the fish species used as peptone source. Underlying these differences, however, the following regularities should be pointed out:

1: Lb 8.01, Lb 10.01 and Lb 3.04, with heterofermentative behaviour, were also the greatest producers of lactic acid (as much as 16.5 g/L), likewise generating high acetic acid values (approximately 7.5 g/L in media with ray and swordfish peptones). On the other hand, these strains consumed practically the entirety of the reducing sugars in all the media. Among these cultures, *L. plantarum* and *L. buchneri* were characterised by the drop in the level of lactic acid, after reaching a maximum, correlative with the increase in concentration of acetic acid.

2: *Leuconostoc mesenteroides*, a typically heterofermentative species, reached very significant collective levels of lactic acid, acetic acid and ethanol in all the media, with clearly sigmoid kinetic profiles, and lacking the discontinuities mentioned in the preceding paragraph.

In quantitative terms, and making use of the models discussed in the preceding section, Table 5 shows the parameters estimated by numerical fitting of the experimental data, together with the yields of the bioproductions. In accordance with *Fisher*'s *F*-test (α=0.05), the equations were consistent in all cases. In accordance with Student's *t*-test (α=0.05), the parameters were significant in 96% of the cases, the remaining 4% always corresponding to the coefficient which translates the lag period. This means simply that in some cultures its value is null, or –which is the same thing– that the beginning of the growth takes place without a period of inoculum acclimatisation.

The individualised analysis of these values, for each LAB and each medium used, allows the following considerations:

Lactobacillus casei **(Lb 3.04):** The waste media led to the highest biomass values (in particular shark peptones: 80% more than the commercial formulation). Besides, this peptone significantly improved the maximum growth rate and, together with that of swordfish, reduced the lag phase for this production. On the other hand, the cfu maximums did not follow these trends. The MRS medium promoted the greatest growths but the corresponding maximum rates were indistinguishable (α=0.05) in the four culture media.

This discrepancy between the biomass values according to their quantification as dry weight or cfu neither is contradictory neither attributable to the experimental error. It simply translates the fact that some media favour the cell division rate and others the production of characteristic metabolites and the accumulation of reserves. On the other

hand, this is of interest from the viewpoint of the metabolites kinetics of different typifications according to the criterion of Luedeking and Piret, as it means that some media are more favourable than others to secondary metabolism.

In the production of organic acids it is possible to distinguish two models: 1) acetic acid reaches very similar maximum levels in all of the media, but its maximum production rate is lower in MRS than in the fish peptones; 2) lactic acid reaches its maximum production in MRS medium and in swordfish peptones, but the maximum production rate appears in ray peptone.

***Lactobacillus plantarum* (Lb 8.01):** The shark proteins –and here also those from ray– led to the highest biomass productions and production rates, though not to the greatest numerical growths. The swordfish peptone yielded similar productions and significantly higher rates than those of MRS. The values of lactic and acetic acids (figure 1 and Table 5) were not treated by means of logistic equation, which is unsuitable due to the aforementioned dynamics of transformation of lactic into acetic acid, which will be covered more fully in a section below.

***Lactococcus lactis* (Lc HD1):** The production of biomass in the marine peptones was clearly higher (260, 190 and 170% in ray, shark and swordfish) than that obtained in MRS. The peptones from chondrichthyes promoted the highest rates, while the highest numerical growth corresponded to MRS. Of all the bacteria assayed, *L. lactis* was the most moderate in the consumption of reducing sugars, and at the same time the lowest producer of lactic acid: approximately 7 g/L in the best cases (MRS and ray), although with higher rates in ray and swordfish. Although the Table 5 shows the parametric

estimations from the fittings corresponding to acetic acid productions, these were very low, of doubtful practical interest from the viewpoint of silage, and the fittings very poor (r=0.867).

***Leuconostoc mesenteroides* (Ln 3.04):** The values of K, v_{mx}, v_{ml}, and v_{mn} were markedly higher in the marine peptones than in MRS. However, this medium led to the highest numerical growth, the highest production of lactic acid and the shortest lag phases for biomass, lactic and acetic acids. In general, the ray peptone promoted the highest yields in all the bioproductions, although the levels of acetic acid were higher in shark peptone and in the commercial media (with no significant differences between these two).

***Pediococcus acidilactici* (Pc 1.02):** The marine peptones significantly (260% in swordfish) surpassed MRS in biomass and production rates, and less markedly in numerical growth and lactic acid. The swordfish peptone induced quick productions of acetic acid (A_m=2.67 g/L, v_{ma}=0.01 $g.L^{-1}.h^{-1}$), at the same time as very low final levels of lactic.

***Lactobacillus buchneri* (Lb 10.01):** The lowest biomasses occurred in MRS and swordfish peptone; the highest in shark peptone. This protein was likewise the promoter of the greatest numerical growth. In this case, together with *Pediococcus* on swordfish peptone, was where the best correlations between the two measurements of biomass occurred. As with *L. plantarum*, the conversion of lactic into acetic acid made impossible the use of logistic equations for the kinetic description of these metabolites. Finally, the media of greatest interest from the viewpoint of their yields indicated highly

disperse directions: shark peptone for biomass, swordfish peptone and MRS for lactic acid, and swordfish peptone for acetic acid.

2: Production of acetic acid in accordance with consumption of lactic acid

We have mentioned in several cases that the productions of lactic and acetic acids were not fitted to logistic models because they did not have the usual sigmoidal profiles which characterise the kinetics of these bioproductions. A plausible explanation would be that the levels of lactic acid in these cases are, as from the beginning of the asymptotic phase of the culture, the result of one active production process and another of consumption or conversion into acetic acid (see figure 1: Lb 8.01).

A possible resource for the fitting of the complete profiles, which yielded highly satisfactory results in the mathematical modelling of the production of bacteriocins by lactic acid bacteria (Cabo et al., 2001b; Vázquez et al., 2005), consists of inserting a term of the type $1–k_L \cdot A(t)$ into the logistic equation corresponding to the lactic acid, and similarly, a term of the type $1–k_A \cdot L(t)$ into that which corresponds to the acetic acid. In this way, we would obtain the following dynamic structures:

$$L = \frac{L_m}{1+\exp\left[2+\frac{4\cdot v_{ml}}{L_m}\cdot\left(\lambda_l - t\right)\right]}\cdot\left[1-k_L\cdot A(t)\right] \qquad (13)$$

$$A = \frac{A_m}{1+\exp\left[2+\frac{4\cdot v_{ma}}{A_m}\cdot\left(\lambda_a - t\right)\right]}\cdot\left[1+k_A\cdot L(t)\right] \qquad (14)$$

However, the values which the numerical solution produces for L_m, v_{ml}, λ_l, A_m, v_{ma} and λ_a do not represent the parameters defined in Table 4, even retaining their original dimensions, but only values of the apparent correlates of these parameters. Thus, the units of the new coefficients k_L and k_A, defined as parameters of proportionality, are in fact [L/g acetic acid] and [L/g lactic acid], respectively. This loss of biological significance has an unfortunate consequence when executing the numerical fitting, as it prevents control of the process by means of the insertion of restrictions which, limiting the values of the parameters between values imposed by the logic of the system, obviate the possibility that the quasi-Newton algorithm may converge at any local minimum of the minimum quadratic function.

A parameter of interest which emerges from this description –the only one, moreover, which retains its physical significance– is the concentration of lactic acid, L_f (or acetic acid, A_f) at the end of the culture (both in g/L). In fact, on calculating the limits of expressions (13) and (14) when $t \to \infty$, one obtains:

$$\lim_{t\to\infty} L = L_f = \frac{L_m}{1+\exp\left[2+\frac{4\cdot v_{ml}}{L_m}\cdot\left(\lambda_l-\infty\right)\right]}\cdot\left[1-k_L\cdot A(\infty)\right] = L_m\cdot\left[1-k_L\cdot A_f\right] \quad (15)$$

$$\lim_{t\to\infty} A = A_f = \frac{A_m}{1+\exp\left[2+\frac{4\cdot v_{ma}}{A_m}\cdot\left(\lambda_a-\infty\right)\right]}\cdot\left[1+k_A\cdot L(\infty)\right] = A_m\cdot\left[1+k_A\cdot L_f\right] \quad (16)$$

expressions which produce, inserting (16) into (15) and finding the value of L_f:

$$L_f = \frac{L_m\cdot\left(1-k_L\cdot A_m\right)}{1+L_m\cdot k_L\cdot k_A\cdot A_m} \quad (17)$$

Similarly, the insertion of (17) into (16) gives rise to the following analytic form:

$$A_f = \frac{A_m \cdot (1 + k_A \cdot L_m)}{1 + L_m \cdot k_L \cdot k_A \cdot A_m} \qquad (18)$$

In short, the model proposed –which, even starting from a structured formulation, represents little more than a quasi-empirical description– is, however, applicable with precision to the forecasting of productive kinetics. Also, it could be useful to interpolation operations, to the formulation of control algorithms which may be incorporated into the software of bioreactors and to the scaling of lactic-acetic fermentation processes.

In figure 2, the bioproductions of lactic and acetic acids are shown, together with their fittings to equations (13) and (14), for one of the "problematic" strains, both on complete and MRS media. In said figure, the satisfactory fitting of the models proposed to experimental reality may be seen. The suitability of said models, at least for the purposes specified above, may be quantified by means of the correlation coefficients between expected and observed results for each case, whose values vary within the interval [0.997-0.999] for lactic acid and [0.992-0.999] for acetic acid.

Among the parameters of greatest descriptive interest, those which possess unequivocal biological significance, that is, L_f and A_f (in g/L), defined respectively by equations (17) and (18), highlighted the following regularities in their numerical values (data not shown):

1: The most significant conversion of lactic into acetic acid by *L. plantarum* occurred in ray peptone (Lb 8.01: L_f =9.50, A_f =3.99), the lowest being in MRS (Lb 8.01: L_f =13.91, A_f =2.36). The values of the proportionality coefficient k_L followed the same trend, that is, in decreasing order: TR > SF > SH > MRS.

2: The same conversion in *Lactobacillus buchneri* cultures obeyed the same decreasing order of importance: shark (L_f =7.77, A_f =7.42), ray (L_f =11.58, A_f =3.70), swordfish (L_f =12.71, A_f =3.78) and MRS medium (L_f =14.47, A_f =2.22). And in this case also, the values of k_L confirmed these results: SH > TR > SF > MRS.

3: Biomass, organic acids, and consumption of nutrients in minimal media.

The next step consisted of reducing as much as possible the nutritive supplements which until now had been added to the marine peptones, with the double goal of reducing costs and bringing the system closer to the conditions of the raw materials which are to be subjected to silage. Therefore, a series of media, which we shall call minimal, were prepared, adding solely glucose to the fish peptones and which, in general, led to lower bioproductions than those obtained in MRS.

Table 5 and figure 3 (as representative example) show the numerical values of the coefficients obtained by adjustment of the kinetic data to the aforementioned equations. All the models were statistically consistent (*Fisher's F*, α=0.05), and 90% of the parameters were significant (*t-Student*, α=0.05), the lag phase once again, for the reasons already discussed, being the parameter whose confidence interval occasionally includes zero. Individualised analysis highlighted the following characteristics:

***Lactobacillus casei* (Lb 3.04):** Although with results which were lower than those obtained with complete media and MRS, the productions were of interest given the schematic of the minimal media. The swordfish peptone promoted the highest values in all parameters.

***Lactobacillus plantarum* (Lb 8.01):** The shark peptone surpassed the remainder in biomass production (although the highest rates occurred in ray and swordfish peptones), with values similar to those of MRS. The highest production of lactic acid occurred in swordfish peptone.

***Lactococcus lactis* (Lc HD1):** As in the complete media, the biomass-related indices were notably better in ray and shark peptones than in MRS. This medium promoted the greatest production of lactic acid, although the highest production rate corresponded to swordfish.

***Leuconostoc mesenteroides* (Ln 3.04):** The absolute productions were generally better in MRS, although the peptones from chondrichthyes –with a particularly long lag period in ray peptone– produced the greatest biomasses. However, the swordfish led the highest rates.

***Pediococcus acidilactici* (Pc 1.02):** The peptones from chondrichthyes –although with considerable lag periods– led to the best biomass-related indices, being particularly notable, the high specific rate (μ_{mn}=8.73 h^{-1}) provided by shark peptone. The highest lactic acid production occurred in MRS.

***Lactobacillus buchneri* (Lb 10.01):** The best results occurred in MRS, being the ray peptone the most productive among the fish peptones.

4: Metabolic typification of lactic and acetic acids, according to the model of Luedeking and Piret

The metabolic characterisation was carried out by fitting the corresponding concentrations to equation (10). The intervals of correlation coefficients between observed and expected values were r=0.935-0.998 (lactic acid) and r=0.810-0.997 (acetic acid). The subsequent application of the Luedeking and Piret criterion to the parametric values of α y β (data not shown) allowed us to conclude that:

1: The lactic acid behaved as a mixed metabolite ($\alpha \neq 0$; $\beta \neq 0$) in 84% of the cultures, and as a primary metabolite ($\alpha \neq 0$; $\beta = 0$) in the remaining 16%.

2: Acetic acid behaved as a mixed metabolite in 17 of the 21 cases in which it was formed, and as a secondary metabolite in the remaining 4.

5: Efficiency clusters and maximum growth rates.

Within the system of species and media studied, the efficiencies in the consumption of proteins might be interpreted as a reflection of highly specific metabolic capabilities. So, it might be possible to distinguish links of a philogenetic type, useful for the selection of groups of species with complementary characteristics, applicable for the

purpose of silage. A confirmative indication of interest for this point of view was found in the cluster analysis of the efficiency matrix itself (Table 6).

In fact, commencing with the complete media (figure 4, first row of hierarchical trees), when Euclidean distances between species in the four-dimensional space of the media are considered, we can observe that the three lactobacilli form a cluster well differentiated from the other three strains. In addition, when the distances between media in the six-dimensional space of the species are considered, we obtain that the peptones from cartilaginous fish (chondrichthyes) are grouped very close together, at a notable distance from the other two (fish with a bony skeleton and meat).

Therefore, this analysis tends to confirm the existence of a peptidic metabolism which is characteristic to lactic acid bacteria. It also suggests significant differences depending on the species or on the protein type, which in principle makes rather improbable the possibility of finding a simple peptidic composition which maximises the bioproductions of the entire group. From the viewpoint of silage, to maintain mixed populations of LAB, an expedient condition for maximising the favourable properties of their action on the substrate (for example: the simultaneous production of high levels of lactic and acetic acids), the use of waste from different species of fish should be considered.

When we change from the complete media to the minimal media (whose most outstanding difference is the absence of the nutrients provided by the yeast extract), the general structure of the clusters of species is maintained (figure 4, second row).

A similar analysis, but based on the biomass production rates (v_{mx} and μ_{mn}) in complete and minimal media (Table 5 and figure 4), allowed the establishment of the following conclusions:

1: In the complete media, the use of the v_{mx} matrix (figure 4, third row) maintains the clusters of two *Lactobacilli*, with a distancing of *L. buchneri* and an approach of *L. lactis*. In the minimal media (figure 4, fourth row) two central nuclei (Lb 8.01-Lb 10.01 and Pc 1.02-Ln 3.04) were generated. On the other hand, the distances between media in the space of the bacteria (on complete and minimal media) retain the arrangement obtained in the case of their efficiencies: a chondrichthyes cluster versus the rest.

2: The use of the μ_{mn} matrix in the complete media (figure 4, fifth row) led to Pc 1.02 joining the initial Lb 8.01-Lb 10.01 group. Strangely, the media tree grouped the shark and swordfish peptones versus the rest. Finally, in the minimal media, the noteworthy value of μ_{mn} in *P. acidilactici* on shark peptone (figure 4, sixth row) caused the distortion of both trees, separating this micro-organism from the rest of the bacteria, and the shark medium from all the others media.

CONCLUSIONS

In general, the peptones from eviscerates of the fishing byproducts –with a slight supplement of yeast extract– showed excellent capability for promoting growth and the characteristic bioproductions (lactic and acetic acids) of the lactic acid bacteria assayed. Given that said micro-organisms form a representative group of useful capabilities for the obtaining of bio-silage, these results allow: 1) the kinetic description of the

microbial growth and biproductions by means of two reparameterized growth models, 2) replacement of the high-cost habitual commercial media in the preparation of the relatively massive starters or inocula required by the process, 3) the definition of the microbiotic combinations of *Lactobacilli* which are expected to be most suitable in order to favour the processes which lead to the bio-silage with the best properties.

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FIGURE CAPTIONS

Figure 1: Cultures of *Lactobacillus plantarum* (Lb 8.01) in MRS medium (●) and in the complete media defined in Table 3 (SF: ○; S: □; TR: ◇). X: biomass; N: colony forming units; ln (N/N_0): Neperian logarithm of the number of cells with regard to the initial value, according to equation (8); L: lactic acid; A: acetic acid; RS: reducing sugars; Pr: proteins. The continuous lines represent the adjustments of the experimental data to the mathematical models described in the text. The corresponding confidence intervals are not shown (α=0.05, n=3), since these didn't transcend in practically any case, the 10% of the experimental mean value.

Figure 2: Lactic acid (●) and acetic acid (○) production kinetics by *L. plantarum* in the media assayed. The continuous lines correspond to the fittings to models (13) and (14). A: SF; B: S; C: TR; D: MRS.

Figure 3: Cultures of *L. buchneri* in MRS (●) and in the minimal media defined in Table 3 (SF: ○; S: □; TR: ◇). Notations as in figure 1.

Figure 4: Hierarchical trees of the LAB in the four-dimensional space of the media (left) and of these in the six-dimensional space of the LAB (right). The hierarchies are based on Euclidean distances, calculated from Tables 5 and 6 in complete (CM) and minimal (MM) media. Note the coherence of the clusters with the factual nature of the axes considered in each case.

FIGURES

FIGURE 1

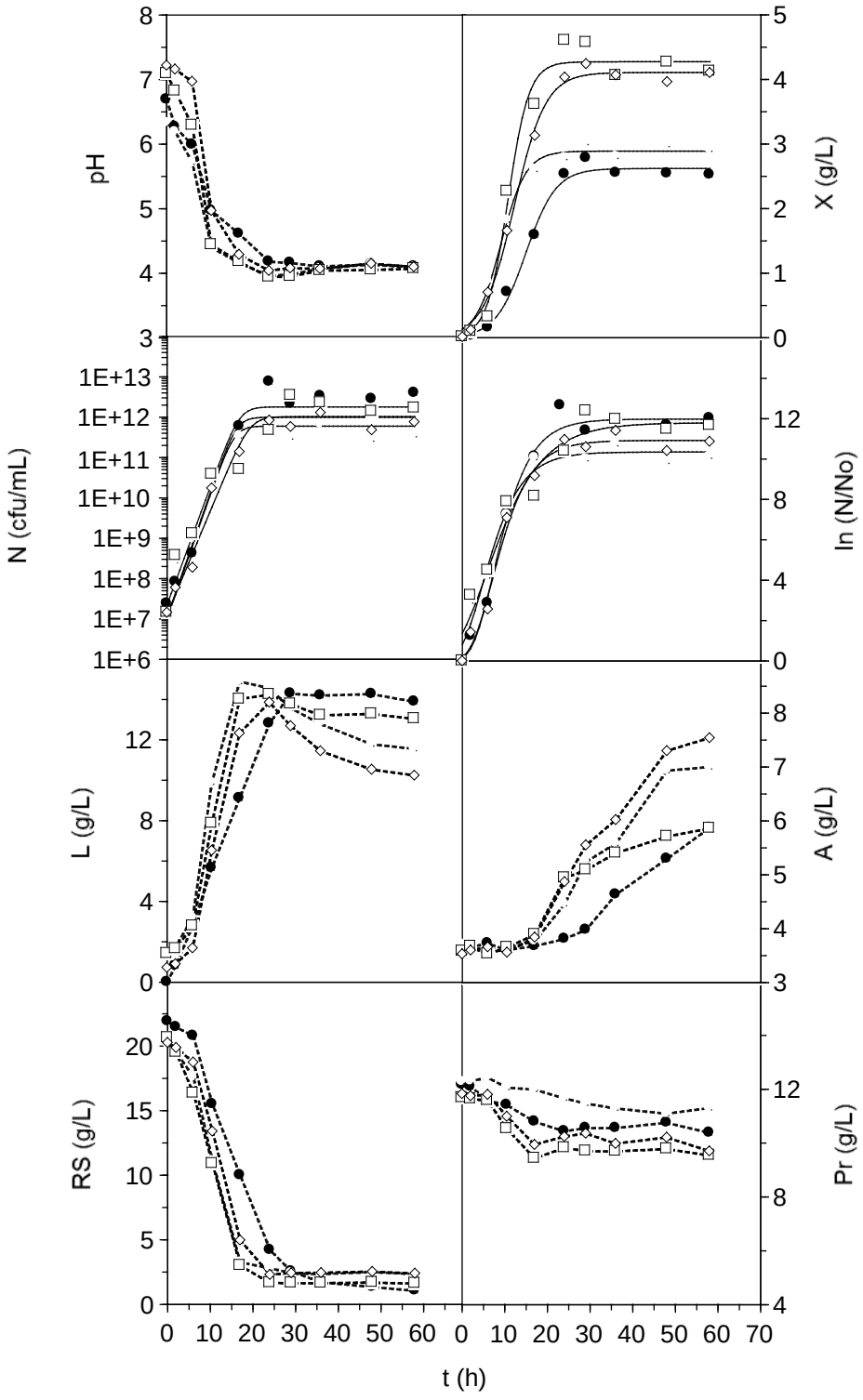


FIGURE 2

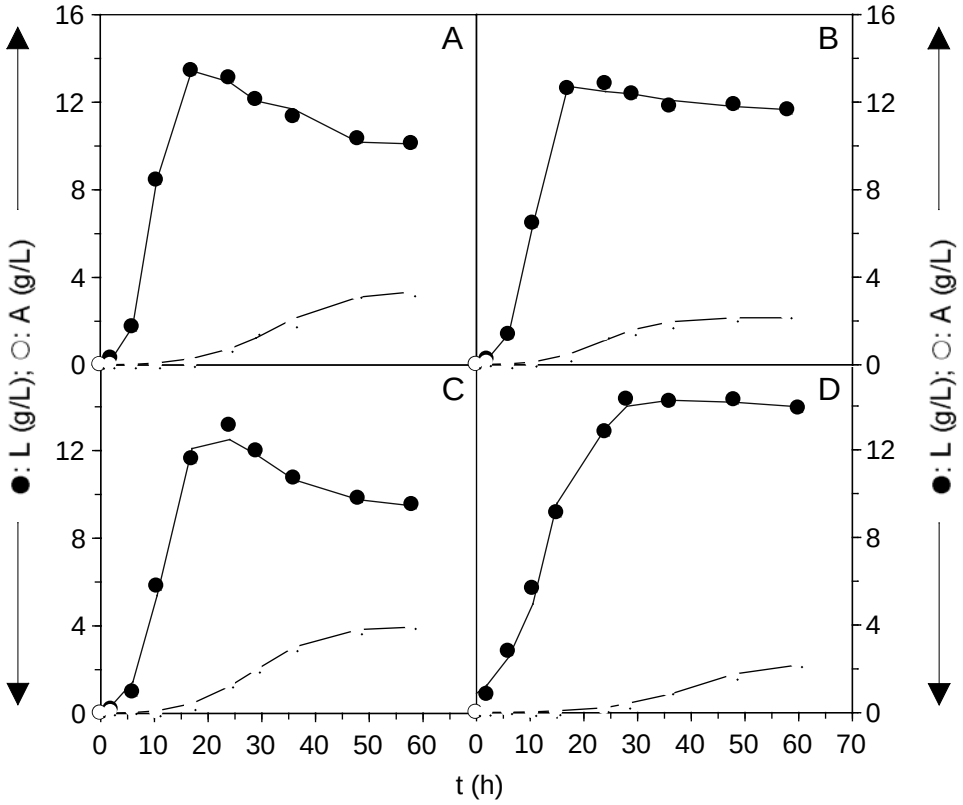

A
B
C
D
●: L (g/L); ○: A (g/L)
●: L (g/L); ○: A (g/L)
t (h)
0
4
8
12
16
0 10 20 30 40 50 60
0 10 20 30 40 50 60 70

FIGURE 3

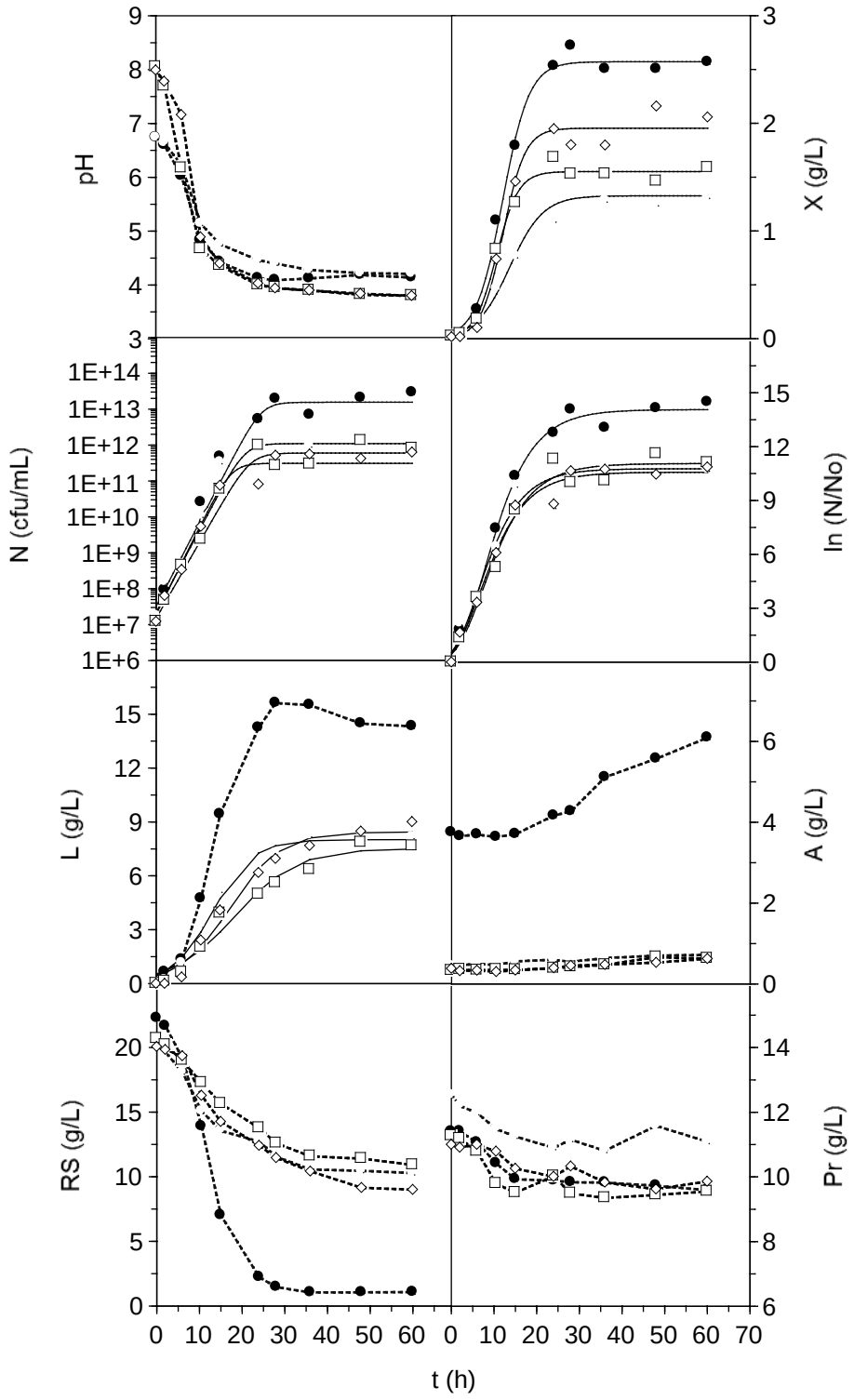
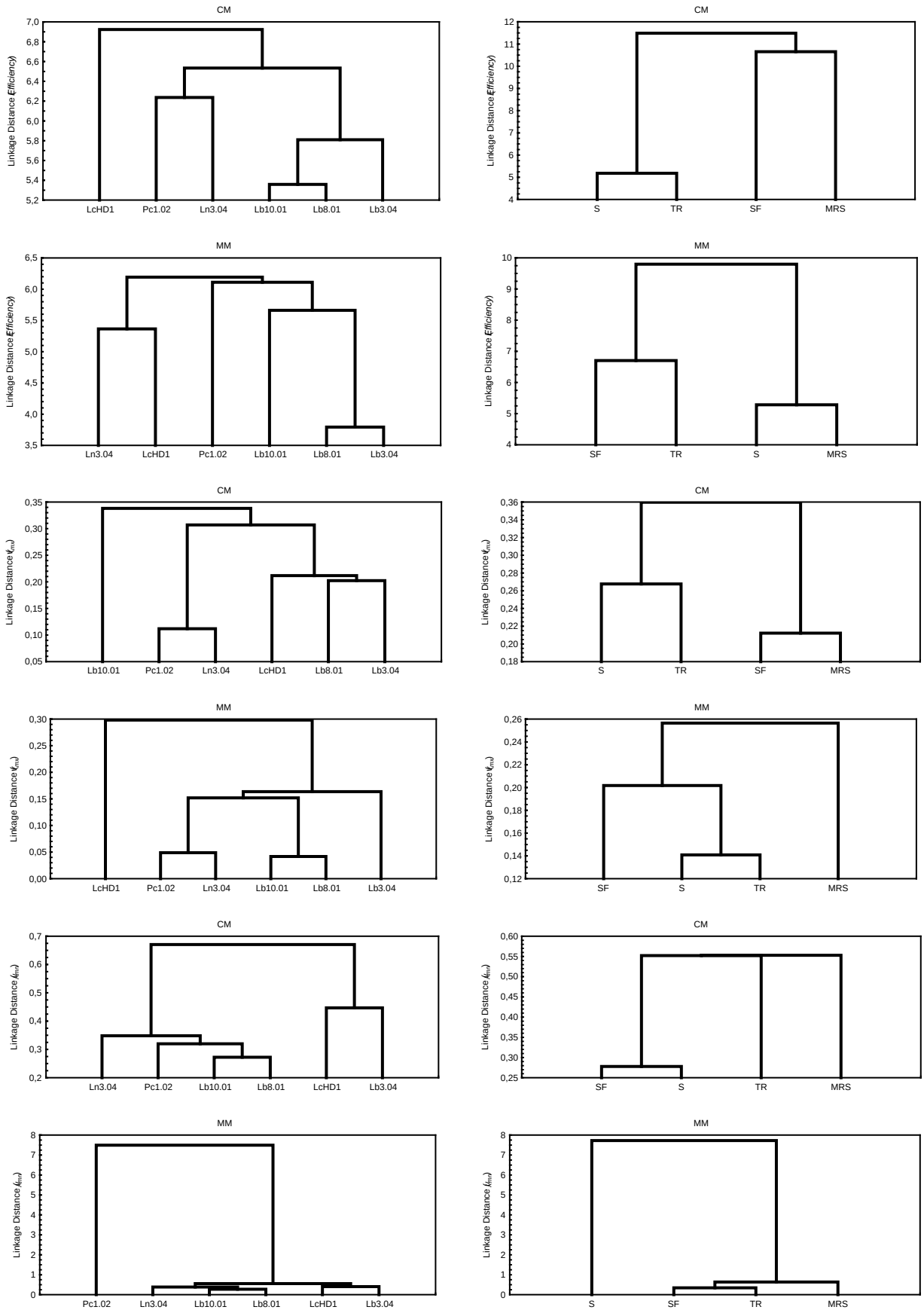


FIGURE 4


CM
Linkage Distance (Efficiency)
7,0
6,8
6,6
6,4
6,2
6,0
5,8
5,6
5,4
5,2
LcHD1
Pc1.02
Ln3.04
Lb10.01
Lb8.01
Lb3.04
CM
Linkage Distance (Efficiency)
12
11
10
9
8
7
6
5
4
S
TR
SF
MRS
MM
Linkage Distance (Efficiency)
6,5
6,0
5,5
5,0
4,5
4,0
3,5
Ln3.04
LcHD1
Pc1.02
Lb10.01
Lb8.01
Lb3.04
MM
Linkage Distance (Efficiency)
10
9
8
7
6
5
4
SF
TR
S
MRS
CM
Linkage Distance (Euclidean)
0,35
0,30
0,25
0,20
0,15
0,10
0,05
Lb10.01
Pc1.02
Ln3.04
LcHD1
Lb8.01
Lb3.04
CM
Linkage Distance (Euclidean)
0,36
0,34
0,32
0,30
0,28
0,26
0,24
0,22
0,20
0,18
S
TR
SF
MRS
MM
Linkage Distance (Euclidean)
0,30
0,25
0,20
0,15
0,10
0,05
0,00
LcHD1
Pc1.02
Ln3.04
Lb10.01
Lb8.01
Lb3.04
MM
Linkage Distance (Euclidean)
0,26
0,24
0,22
0,20
0,18
0,16
0,14
0,12
SF
S
TR
MRS
CM
Linkage Distance (Manhattan)
0,7
0,6
0,5
0,4
0,3
0,2
Ln3.04
Pc1.02
Lb10.01
Lb8.01
LcHD1
Lb3.04
CM
Linkage Distance (Manhattan)
0,60
0,55
0,50
0,45
0,40
0,35
0,30
0,25
SF
S
TR
MRS
MM
Linkage Distance (Manhattan)
8
7
6
5
4
3
2
1
0
Pc1.02
Ln3.04
Lb10.01
Lb8.01
LcHD1
Lb3.04
MM
Linkage Distance (Manhattan)
8
7
6
5
4
3
2
1
0
S
SF
TR
MRS

TABLES

Table 1. Lactic acid bacteria used.

Strains	References	Key IIM
Lactobacillus plantarum	CECT 220	Lb 8.01
Lactobacillus buchneri	CECT 4111	Lb 10.01
Lactobacillus casei ssp. casei	CECT 4043	Lb 3.04
Lactococcus lactis ssp. lactis	HD1-IIM	Lc HD1
Leuconostoc mesenteroides ssp. mesenteroides	CECT 219	Ln 3.04
Pediococcus acidilactici	NRLL B-5627	Pc 1.02

CECT: Spanish Type Culture Collection (University of Valencia, Spain).
NRRL: Northern Regional Research Laboratory (Peoria, Illinois, USA).
HD-IIM: Department Animal Science, University of Wyoming (Wyoming, USA)
Key IIM: Abbreviated notation used in this work.

Table 2. Main composition (g/L) of fish peptones (FP) from fish viscera. SF: sword fish; S: shark; TR: thornback ray.

	Proteins (Lowry)	Total sugars	Total nitrogen
SF	18.7	0.8	3.8
S	31.3	2.2	10.7
TR	22.0	1.1	8.2

Table 3. Composition of culture media used in microbiological kinetics (g/L).

	CM media [1]	MM media[1]	MRS medium
Glucose	20.00	20.00	20.00
Yeast extract	4.00	-	4.00
Sodium acetate	5.00	-	5.00
Ammonium citrate	2.00	-	2.00
K_2HPO_4	2.00	-	2.00
$MgSO_4$	0.20	-	0.20
$MnSO_4$	0.05	-	0.05
Tween 80	-	-	1.00
Meat extract	-	-	8.00
Bactopeptone	-	-	10.00
Fish peptone[1] protein (Lowry)	10.00	10.00	-

CM: Complete media.
MM: Minimum media.
([1]): The three types of fish peptones defined in Table 2.

Table 4. Symbolic notations used.

r_x :	Growth rate (biomass production). Dimensions: $g.L^{-1}.h^{-1}$
X :	Biomass. Dimensions: g/L
t :	Time. Dimensions: h
K :	Maximum biomass. Dimensions: g/L
μ_{mx} :	Specific maximum growth rate (biomass production per unit of biomass and time). Dimensions: h^{-1}
X_0 :	Initial biomass. Dimensions: g/L
v_{mx} :	Maximum growth rate (biomass production). Dimensions: $g.L^{-1}.h^{-1}$
λ_x :	Growth lag phase (biomass production). Dimensions: h
L :	Lactic acid. Dimensions: g/L
L_m :	Maximum lactic acid. Dimensions: g/L
v_{ml} :	Maximum lactic acid rate production. Dimensions: $g.l^{-1}.h^{-1}$
λ_l :	Lactic acid lag phase. Dimensions: h
A :	Acetic acid. Dimensions: g/L
A_m :	Maximum acetic acid. Dimensions: g/L
v_{ma} :	Maximum acetic acid rate production. Dimensions: $g.l^{-1}.h^{-1}$
λ_a :	Acetic acid lag phase. Dimensions: h
N :	Microorganism number per volume. Dimensions: cfu/mL
N_0 :	Initial microorganism number per volume. Dimensions: cfu/mL
μ_{mn} :	Specific maximum growth rate (micoorganism production). Dimensions: h^{-1}
N_m :	Maximum growth (micoorganism production). Dimensions: cfu/mL
v_{mn} :	Maximum growth rate (micoorganism production). Dimensions: $cfu.mL^{-1}.h^{-1}$
λ_n :	Growth lag phase (initial time of growth). Dimensions: h
r_p :	Production rate for product (X, L, A). Dimensions: $g.L^{-1}.h^{-1}$
α :	Luedeking and Piret parameter (growth-associated constant for product formation). Dimensions: g product / g biomass
β :	Luedeking and Piret parameter (non growth-associated constant for product formation). Dimensions: $g\ product.g^{-1}(biomass).h^{-1}$
P :	Product concentration (X, L, A) formed by the microorganism. Dimensions: g/L
RS :	Reducing sugars concentration consumed by the microorganism. Dimensions: g/L
Pr :	Protein concentration consumed by the microorganism. Dimensions: g/L
$Y_{P/Pr}$	Product formation / protein consumption. Dimensions: g product or BU / g protein
$Y_{P/RS}$	Product formation / reducing sugars consumption. Dimensions: g product or BU / g reducing sugars

Table 5. Main parametric estimations (defined in the table 4) of the kinetic models (3, 8-9, 11-12; see text), describing the bioproductions of biomass, lactic and acetic acids by the lactic acid bacteria that are summarized in the table 1, as well as their corresponding growths and productive yields, in the complete specified media (CM, table 3) and minimum specified media (MM, table 3). Intervals of correlation coefficients among observed and expected productions were: *X*: r=0.924-0.999; *L*: r=0.947-1.000; *A*: r=0.867-0.998; ln (N/N_0): r=0.967-0.999.

	MEDIA	BIOMASS (*X*)			LACTIC (*L*)			ACETIC (*A*)			GROWTH [ln (N/N_0)]			YIELDS (*Y*)					
		K	v_{mx}	λ_x	L_m	v_{ml}	λ_l	A_m	v_{ma}	λ_a	B	μ_{mn}	N_m	$Y_{X/Pr}$	$Y_{X/AR}$	$Y_{L/Pr}$	$Y_{L/AR}$	$Y_{A/Pr}$	$Y_{A/AR}$
Lb 3.04	MRS	2.88	0.37	5.9	14.0	1.01	3.0	2.67	0.07	10.0	14.1	1.06	4.9×10^{13}	1.6	0.12	8.2	0.61	1.45	0.11
	TR-CM	3.33	0.37	7.3	11.9	1.64	8.1	2.41	0.10	8.1	12.4	1.18	1.2×10^{13}	1.8	0.17	6.6	0.60	1.39	0.13
	TR-MM	1.83	0.17	6.9	6.31	0.26	4.3	1.35	0.27	10.9	11.0	0.84	2.4×10^{12}	2.2	0.25	7.6	0.86	1.56	0.18
	S-CM	4.82	0.46	4.7	11.5	1.01	5.3	2.48	0.13	9.1	12.6	1.02	1.7×10^{13}	2.5	0.22	6.1	0.54	1.44	0.13
	S-MM	1.78	0.17	6.2	6.56	0.29	4.4	0.94	0.13	17.5	12.4	1.02	9.4×10^{12}	1.2	0.16	4.5	0.58	0.53	0.07
	SF-CM	3.38	0.26	4.3	12.8	1.48	6.3	2.86	0.11	4.7	12.4	1.05	1.2×10^{13}	2.8	0.15	10.9	0.61	2.48	0.14
	SF-MM	2.37	0.11	-4.0	7.68	0.65	4.5	1.31	0.03	-2.3	12.5	0.95	1.1×10^{13}	2.9	0.28	10.6	1.02	1.49	0.14
Lb 8.01	MRS	2.62	0.19	7.8	-	-	-	-	-	-	12.0	0.90	3.8×10^{12}	1.4	0.12	7.8	0.67	1.25	0.11
	TR-CM	4.11	0.28	4.9	-	-	-	-	-	-	10.9	0.80	8.0×10^{11}	1.9	0.23	4.5	0.53	1.88	0.23
	TR-MM	1.88	0.18	10.9	7.96	0.31	7.2	-	-	-	11.0	0.58	1.1×10^{12}	2.0	0.19	8.6	0.82	0.27	0.03
	S-CM	4.27	0.44	5.8	-	-	-	-	-	-	11.8	0.59	1.9×10^{12}	1.9	0.22	5.4	0.61	1.07	0.12
	S-MM	2.34	0.19	10.6	7.68	0.28	4.3	-	-	-	12.5	0.71	4.7×10^{12}	1.7	0.23	5.6	0.77	-	-
	SF-CM	2.89	0.25	3.1	-	-	-	-	-	-	10.4	0.75	4.5×10^{11}	3.0	0.16	10.3	0.57	3.36	0.19
	SF-MM	1.45	0.08	6.3	8.53	0.53	6.8	-	-	-	9.9	0.44	5.8×10^{11}	1.3	0.13	7.6	0.79	0.25	0.03
Lc HD1	MRS	1.05	0.17	3.5	7.0	0.66	2.1	-	-	-	10.1	1.31	2.0×10^{11}	1.1	0.09	8.3	0.69	*0.13*	*0.01*
	TR-CM	2.65	0.37	3.0	6.3	1.07	4.1	0.43	0.04	1.9	8.7	1.53	8.1×10^{10}	1.3	0.23	3.4	0.61	0.22	0.04
	TR-MM	2.02	0.33	5.6	3.71	0.58	3.5	-	-	-	9.0	1.03	5.7×10^{10}	2.0	0.34	3.9	0.66	0.03	0.01
	S-CM	1.95	0.27	3.2	7.0	0.92	3.2	0.35	0.03	8.6	8.0	1.13	3.9×10^{10}	1.3	0.19	4.9	0.74	0.23	0.04
	S-MM	2.25	0.40	7.3	3.61	0.32	1.7	-	-	-	10.1	1.25	1.6×10^{11}	1.7	0.34	2.9	0.58	0.24	0.05
	SF-CM	1.72	0.15	2.0	6.1	0.54	1.7	0.36	0.09	9.2	8.4	1.07	5.6×10^{10}	1.9	0.21	7.3	0.80	0.37	0.04
	SF-MM	1.07	0.22	8.4	4.87	0.81	6.2	-	-	-	9.7	1.05	1.5×10^{11}	1.0	0.12	4.9	0.59	0.27	0.03
Ln 3.04	MRS	0.69	0.08	6.3	8.8	0.42	3.8	3.34	0.14	8.4	8.9	0.60	1.6×10^{11}	0.8	0.03	10.2	0.43	4.05	0.17
	TR-CM	1.31	0.11	23.6	6.6	0.54	23.8	2.89	0.11	17.3	7.7	0.64	9.8×10^{9}	2.5	0.11	9.5	0.40	4.38	0.18
	TR-MM	1.29	0.07	19.9	4.86	0.13	23.5	3.32	0.09	24.6	4.8	0.35	2.7×10^{9}	0.7	0.20	1.9	0.51	1.18	0.32
	S-CM	2.05	0.16	13.4	7.3	0.66	16.6	3.31	0.19	14.4	8.0	0.93	1.3×10^{10}	1.2	0.07	6.1	0.36	2.61	0.16
	S-MM	1.89	0.15	9.2	4.53	0.18	3.3	3.29	0.11	6.2	7.6	0.75	4.4×10^{10}	1.0	0.21	2.2	0.49	1.34	0.29
	SF-CM	1.40	0.10	12.8	7.3	0.54	14.4	2.81	0.18	15.2	7.7	0.78	1.0×10^{10}	0.9	0.08	5.1	0.41	2.05	0.16
	SF-MM	0.69	0.07	7.8	6.17	0.51	8.9	2.60	0.19	11.3	7.4	0.64	3.3×10^{10}	0.6	0.06	5.6	0.57	2.00	0.21
Pc 1.02	MRS	1.17	0.06	8.0	7.9	0.32	5.6	0.97	0.04	22.9	11.8	1.01	8.0×10^{11}	1.1	0.10	7.4	0.67	0.90	0.08
	TR-CM	2.04	0.08	7.7	10.2	0.29	4.0	0.53	0.04	15.6	11.3	0.43	9.8×10^{11}	1.3	0.14	6.7	0.70	0.37	0.04
	TR-MM	1.64	0.09	23.4	4.76	0.21	21.1	-	-	-	8.8	0.67	4.3×10^{10}	1.8	0.23	5.7	0.72	0.21	0.03
	S-CM	2.27	0.09	4.6	10.0	0.30	3.1	0.53	0.02	7.1	11.6	0.67	1.3×10^{12}	1.4	0.17	6.5	0.75	0.33	0.04
	S-MM	1.32	0.19	19.3	4.61	0.16	13.2	-	-	-	10.3	8.73	1.9×10^{11}	1.0	0.22	3.3	0.75	-	-
	SF-CM	2.86	0.18	5.3	9.5	0.45	5.1	2.67	0.10	19.9	11.8	0.64	1.6×10^{12}	1.8	0.15	5.8	0.49	1.58	0.14
	SF-MM	1.28	0.06	7.4	5.31	0.20	6.4	-	-	-	9.2	0.71	2.2×10^{11}	0.8	0.16	3.5	0.69	0.12	0.02
Lb 10.01	MRS	2.57	0.21	5.7	-	-	-	-	-	-	14.1	0.82	1.9×10^{13}	1.4	0.12	7.9	0.68	1.29	0.11
	TR-CM	3.32	0.66	9.9	-	-	-	-	-	-	13.6	0.67	1.7×10^{13}	1.4	0.17	4.7	0.59	1.41	0.18
	TR-MM	1.95	0.19	6.9	8.44	0.36	5.6	-	-	-	10.6	0.64	4.8×10^{11}	1.8	0.18	7.9	0.81	0.21	0.02
	S-CM	4.03	0.50	8.3	-	-	-	-	-	-	15.0	0.75	6.7×10^{13}	1.9	0.20	5.6	0.60	1.41	0.15
	S-MM	1.55	0.15	5.4	7.52	0.26	4.0	-	-	-	11.1	0.64	7.7×10^{11}	0.9	0.16	4.5	0.78	0.17	0.03
	SF-CM	2.34	0.33	8.7	-	-	-	-	-	-	15.0	0.59	5.0×10^{13}	1.6	0.13	7.9	0.66	2.24	0.19
	SF-MM	1.33	0.09	5.8	8.02	0.42	3.8	-	-	-	10.8	0.69	8.5×10^{11}	0.9	0.13	5.6	0.81	0.19	0.03

Table 6. Matrix of efficiencies in protein consumptions (initial level × 100 / final level) for all the combinations of media (CM: complete media and MM: minimum media) and lactic acid bacteria used.

CM	Lb 3.04	Lb 8.01	Lb 10.01	Lc HD1	Ln 3.04	Pc 1.02
MRS	85.20	85.29	84.12	92.75	92.30	89.71
TR	86.21	81.99	80.17	84.11	84.45	87.72
S	85.50	81.68	82.56	88.30	82.79	87.22
SF	90.84	92.02	87.20	92.25	88.09	86.70
MM						
MRS	85.20	85.29	84.12	92.75	92.30	89.71
TR	91.76	92.37	89.64	92.01	91.27	93.23
S	86.14	89.33	84.74	90.00	93.42	88.47
SF	93.38	91.42	88.44	93.26	89.21	87.38