Mathematical tools for objective comparison of microbial cultures. Application to evaluation of 15 peptones for lactic acid bacteria productions.

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Headline: bacteriocins production on waste and commercial media.

Keywords: logistic equation, lactic acid bacteria, MRS media, commercial peptones, fish viscera peptones, lactic acid, nisin, pediocin.

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ABSTRACT

Using the logistic model as a starting point, a set of reparameterised equations were established which permit the easy calculation of the relevant parameters of microbial kinetics, together with their confidence limits, with the aim of establishing rigorous comparison between cultures under differing conditions. When the resource was used to evaluate the aptitude of peptones from diverse sources for the culture of lactic acid bacteria (LAB), a great variability was found, even among the results of commercial formulations with the same denomination. None of the peptones was able to maximise the growth –very active in fish peptones– and the production of the characteristic metabolites at the same time. Under these conditions, the application of the cluster analysis to kinetic parameters of proven descriptive capability becomes a useful exploratory method, which allows to decide combinations of protein sources apt to make compatible different potential purposes of LAB cultures. This way, it is possible to design factorial experiments to search optimum values for these purposes on the basis of hypothesis which avoid the selection of superfluous variables and inadequate domains.

INTRODUCTION

From the point of view of their industrial interest, lactic acid bacteria (LAB) are an important microbial group, due to their role in food fermentation and preservation, either as natural microbiota, or inocula added under controlled conditions. Among the bioactive molecules produced by LAB are lactic acid, acetic acid, ethanol, diacetyl, 2,3-butanediol and bacteriocins [1, 2]. Bacteriocins are peptides with antimicrobial activity and have interest in alimentary industry as they are innocuous, sensitive to digestive proteases, and do not change the organoleptic properties of the food [3, 4]. However, the large-scale production of LAB and bacteriocins is expensive due to the complex media –rich in protein hydrolysates– which they require for growth. Commercial media as MRS, TGE or APT solve the problem of protein sources, by means of products such as bactopectone, tryptone, meat extract or yeast extract (sometimes all of them) in formulations which, however, reach high costs.

Even if these peptones are necessary for bacteriocin production [5-7], the efficiencies (substrate consumed/initial substrate) of these media are usually low, suggesting unbalanced proportions of nutrients [8]. Thus, the protein materials which remain in the media at the end of the incubation constitute superfluous expenditure and hinder the subsequent purification of the bacteriocins. The replacement of these proteins by inorganic sources of nitrogen does not produce acceptable results [9], nor is suitable the initially obvious solution of adjusting the initial protein level to the detected consumption [5]. It is this way because peptones do not represent simply a source of organic nitrogen, but rather a source of amino acids or peptides with specific roles, in such a way that only a fraction of the total added is really important [10-15]. So, the use of low-cost protein fractions will bring about a reduction in large-scale production costs. Furthermore, if food waste (as that generated by the processing of resources from marine origin) is used to obtain those protein fractions, a productive cycle is closed: recycling of a pollutant waste and obtaining products (bacteriocins) with high added value, useful for preservation of foodstuffs.

According to the definition of Green et al. [16], ‘peptones’ are water-soluble protein hydrolysates non-coagulable by heat. Commercial peptones used in microbiological media are mainly derived from casein, soy and meat. Peptones from marine origin are barely used today, in spite of their good results in some applications, as is the case with the production of proteases by *Bacillus subtilis* [17] or *Vibrio* species [18], with gastrine and epidermal growth factor (EGF) by mouse fibroblasts [19], glycerol by *Saccharomyces cerevisiae* [20], lactic acid bacteria [21-23], probiotic marine bacteria [24], bacteriocins [25, 26] or microbial growth [27, 28].

This study attempts to validate the use, in more general terms, of fish peptones, through an approximation which presents the following characteristics. 1) The study includes a comparison, from various angles, between the results obtained with 4 ‘marine’ peptones specifically prepared for this purpose and 11 commercial formulations. 2) Microorganisms used were LAB, well known for a complex nutritional requirements which demands diversified peptide sources. 3) Comparisons were performed in the most rigorous way possible, using parametric estimations with biological significance and verified statistic reliability, obtained through the fitting of all the cases studied to the

same mathematical models, whose pertinence was discussed at a formal level and verified through experimental results.

MATERIALS AND METHODS

Preparation of marine peptones from fish viscera

Raw materials used were viscera from swordfish (*Xiphias gladius*), shark (*Isurus oxyrinchus*), thornback ray (*Raja clavata*) and yellowfin tuna (*Thunnus albacares*), sampled immediately after industrial processing and maintained at -20°C until use. Storage did not exceed 15 days for all the materials. Visceral masses (stomach and intestine) were ground with equal weights of distilled water, and the homogenates, after steam flow stabilisation ($101^{\circ}\text{C}/1$ hour), were treated in a centrifuge decanter at $7,500\times g/15$ minutes [25, 26]. Supernatants, or marine peptones, were typified by determining the levels of total nitrogen, protein and total sugars, and stored at -20°C until time of use for the formulation of culture media. Raw composition of these peptones is shown in Table 1.

Microbiological methods

Microorganisms used as bacteriocin producers were *Pediococcus acidilactici* NRRL B-5627 (abbreviated key Pc 1.02), from Northern Regional Research Laboratory (Peoria, IL, USA), and *Lactococcus lactis* subsp. *lactis* (abbreviated key Lc HD1) isolated from salmon sausages and supplied by Dr. López Cabo (IIM-CSIC, Spain). *Carnobacterium piscicola* CECT 4020 (Spanish Type Culture Collection) and *Leuconostoc mesenteroides* subsp. *lysis* (kindly provided by Dr. Ray, University of Wyoming, Laramie, USA) were used as indicators in bacteriocin bioassays. Stock cultures were stored at -75°C in MRS medium (Pronadisa, Hispanlab S.A., Spain) with 25% glycerol [8]. Inocula (1% v/v) consisted of cellular suspensions from 24-hour (Pc 1.02) and 12-hour (Lc HD1) cultures on MRS medium, adjusted to an OD ($\lambda=700$ nm) of 0.900.

The commercial protein sources here studied (Table 2) were always used at a concentration equivalent to that determined (Lowry) in MRS medium. In all cases, the initial pH was adjusted to 7.0 and the solutions sterilized at 121°C, 15 min. Microorganisms were grown at 30°C in 300 mL Erlenmeyer flasks with 200 mL of medium (optimal conditions for nisin and pediocin productions [29]), under orbital shaking at 200 rpm. Cultures were carried out in triplicate.

Analytical methods

At pre-established times, each culture was divided into two aliquots. The first one was centrifuged at 4,000×g for 15 minutes, the sediment washed twice and resuspended in distilled water to an appropriate dilution for OD measuring at 700 nm. Dry weight can then be estimated from a previous calibration curve. The corresponding supernatant was used for determination of proteins, lactic and acetic acids, and reducing sugars. The second aliquot was used for extraction and quantification of pediocin (produced by Pc 1.02) and nisin (produced by Lc HD1), using *C. piscicola* and *L. mesenteroides*, respectively, as indicators, according to previously described methods [30, 31].

Other analytical determinations were: *Total nitrogen*: method of Havilah et al. [32], applied to digests obtained through the classic Kjeldahl procedure. *Proteins*: method of Lowry et al. [33]. *Total sugars*: phenol-sulphuric reaction [34] according to the application of Strickland and Parsons [35] with glucose as a standard. *Reducing sugars*: 3,5-dinitrosalicylic method [36]. *Lactic and acetic acids*: HPLC, after membrane filtration of samples (0.22 µm Millex-GV, Millipore, USA), using an ION-300 column (Transgenomic, USA) with 6 mM sulphuric acid as a mobile phase (flow=0.4 mL/min) at 65°C and a refractive-index detector. All assays were carried out in duplicate.

Mathematical models

A widely accepted model for the macroscopic description of the microbial growth kinetics is the logistic equation [37-40], one advantage of which is the direct biological significance of its parameters. This model describes the biomass variation against time

by means of the following differential equation, typical for an auto-catalytic mechanism (see notations in Table 3):

$$r_X = \frac{dX}{dt} = \mu_{mX} \cdot X \cdot \left(\frac{K - X}{K} \right) \quad (1)$$

which, integrated between $X_0 \rightarrow X$ and $0 \rightarrow t$, produces the explicit expression:

$$X = \frac{K}{1 + \exp(c - \mu_{mX} \cdot t)}, \text{ with } c = \ln\left(\frac{K}{X_0} - 1\right) \quad (2)$$

Besides those included in (2), another parameter of interest [18, 41], robust in the sense that it is not very sensitive to experimental error and hence specially useful for comparative purposes, is the maximum growth rate (v_{mX}), or slope of the straight tangent to the function at its inflection point (t_i). Taking the second derivative to zero and isolating the abscissa of the inflection point ($t=t_i$), we obtain:

$$\frac{d^2 X}{dt^2} = \frac{-\mu_{mX}^2 \cdot K \cdot e^{c - \mu_{mX} \cdot t} \cdot (1 + e^{c - \mu_{mX} \cdot t})^2 + K \cdot \mu_{mX}^2 \cdot e^{2(c - \mu_{mX} \cdot t)} \cdot 2 \cdot (1 + e^{c - \mu_{mX} \cdot t})}{(1 + e^{c - \mu_{mX} \cdot t})^4} \quad (3)$$

$$\frac{d^2 X}{dt^2} = 0 \xrightarrow{t=t_i} -(1 + e^{c - \mu_{mX} \cdot t_i}) + 2 \cdot e^{c - \mu_{mX} \cdot t_i} = 0$$

$$\Rightarrow t_i = \frac{c}{\mu_{mX}} \quad (4)$$

from which the value of the slope (v_{mX}) is:

$$v_{mX} = \left(\frac{dX}{dt} \right)_{t_i} = \frac{K \cdot \mu_{mX} \cdot e^{c - \mu_{mX} \cdot t_i}}{(1 + e^{c - \mu_{mX} \cdot t_i})^2} = \frac{K \cdot \mu_{mX} \cdot e^{c - \mu_{mX} \cdot \frac{c}{\mu_{mX}}}}{\left(1 + e^{c - \mu_{mX} \cdot \frac{c}{\mu_{mX}}} \right)^2} = \frac{K \cdot \mu_{mX}}{4} \quad (5)$$

Taking into account the geometry of the function, we can obtain an analytical expression for the lag phase of the culture (λ_X), defined as the intersection of the tangent at the inflection point with the abscissa axis. Therefore the value for the biomass when $t=t_i$ is:

$$X(t_i) = X \Big|_{t=t_i} = \frac{K}{1 + \exp\left(c - \mu_{mX} \cdot \frac{c}{\mu_{mX}}\right)} = \frac{K}{1 + e^0} = \frac{K}{2}$$

and the equation of that tangent:

$$R = X(t_i) + f'(t_i) \cdot (t - t_i) = X(t_i) + \left(\frac{dX}{dt}\right)_{t_i} \cdot (t - t_i) = \frac{K}{2} + v_{mX} \cdot (t - t_i) \quad (6)$$

Therefore, the value for λ_X , or time (t) when $R=0$, is:

$$\frac{K}{2} + v_{mX} \cdot (\lambda_X - t_i) = 0 \Rightarrow \lambda_X = \frac{v_{mX} \cdot t_i - \frac{K}{2}}{v_{mX}} = \frac{\frac{K \cdot \mu_{mX}}{4} \cdot \frac{c}{\mu_{mX}} - \frac{K}{2}}{\frac{K \cdot \mu_{mX}}{4}} = \frac{c - 2}{\mu_{mX}} \quad (7)$$

In order to compare microbial kinetics it is important to use expressions –re-parameterised if necessary– in which the coefficients with biological significance appear explicitly. This facilitates the necessary definition of the confidence limits for their estimates using informatic applications. To comply with this need, in our case the equations (5) and (7) must be introduced into (2), which leads us to the definitive expression:

$$X = \frac{K}{1 + \exp(c - \mu_{mX} \cdot t)} \xleftrightarrow[c=2 + \frac{4 \cdot v_{mX} \cdot \lambda}{K}]{\mu_{mX} = \frac{4 \cdot v_{mX}}{K}} X = \frac{K}{1 + \exp\left[2 + \frac{4 \cdot v_{mX}}{K} \cdot (\lambda_X - t)\right]} \quad (8)$$

The same operations may be also applied to other aspects of microbial kinetics, introducing the corresponding dependent variables into the equation (1) and redefining

the parameters μ_{mX} , K and λ_X . In this way, the production of lactic acid (L), acetic acid (A) and bacteriocin (BT) can be described by means of the following equations:

$$L = \frac{L_m}{1 + \exp \left[2 + \frac{4 \cdot v_{mL}}{L_m} \cdot (\lambda_L - t) \right]} \quad (9)$$

$$A = \frac{A_m}{1 + \exp \left[2 + \frac{4 \cdot v_{mA}}{A_m} \cdot (\lambda_A - t) \right]} \quad (10)$$

$$BT = \frac{BT_m}{1 + \exp \left[2 + \frac{4 \cdot v_{mBT}}{BT_m} \cdot (\lambda_{BT} - t) \right]} \quad (11)$$

On the other hand, in order to typify the nature of the microbial metabolites, we used the criteria of Luedeking and Piret [42], based on the relationship between the growth rate and the production of the particular metabolite in which we are interested. This way, we have:

$$r_p = \alpha \cdot \frac{dX}{dt} + \beta \cdot X = \alpha \cdot r_X + \beta \cdot X \quad (12)$$

which permits us to classify an particular metabolite as primary, secondary and mixed depending on its production rate is, respectively, a function of the growth rate ($\alpha \neq 0$; $\beta = 0$), the present biomass ($\alpha = 0$; $\beta \neq 0$), or both magnitudes ($\alpha \neq 0$; $\beta \neq 0$).

Finally, in all cases we calculated the production yield (of biomass or metabolites) with respect to a nutrient, as the relationship between the production level and the nutrient consumed in a given time interval (usually the total duration of the culture). Yields of biomass (X), lactic acid (L), acetic acid (A) and bacteriocins (BT) were referred to the consumptions of both reducing sugar and protein, and quantified as (see Table of symbolic notations):

$$Y_{P/RS} = \frac{\Delta P}{\Delta RS} = \frac{P_f - P_i}{RS_i - RS_f} \quad (13)$$

$$Y_{P/Pr} = \frac{\Delta P}{\Delta Pr} = \frac{P_f - P_i}{Pr_i - Pr_f} \quad (14)$$

In the case of the bacteriocins the yield with respect to the biomass was also calculated, which allowed us to define specific productivity values under different conditions:

$$Y_{BT/X} = \frac{\Delta BT}{\Delta X} = \frac{BT_f - BT_i}{X_f - X_i} \quad (15)$$

Numerical 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 spreadsheet. Statistica 6.0 (StatSoft, Inc. 2001) and Simfit 5.6.7 (University of Manchester, UK) programs were used to evaluate the significance of the parametric estimates (Student’s *t* test, $\alpha=0.05$), the consistency of the models (Fisher’s *F* test, $\alpha=0.05$), and to perform the cluster analysis.

RESULTS AND DISCUSSION

In accordance with the focus of this study, the experimental plan involved the obtention of the kinetic data necessary for the description of Pc 1.02 and Lc HD1 cultures in the media specified in Table 2, using the equations described in the section of methods. The variables measured were: pH, biomass production (dry weight), consumption of reducing sugars and proteins, production of lactic acid, acetic acid and bacteriocins (pediocin and nisin). Since the MRS medium contains acetic acid, and the inocula, as well as marine peptones, necessarily include low, but detectable, levels of organic acids (and in some cases bacteriocins), the initial concentrations of these components were subtracted from the analytical values. This was done not only in order to obtain strict net production

values, but also to avoid possible artificial biases in the parametric estimates, due to the existence of a non-null intercept.

*Production of biomass, organic acids and pediocin by *P. acidilactici**

Figure 1 shows the experimental results of the Pc 1.02 cultures, in which the time-course of the main variables in the different protein sources can be appreciated. Parametric estimates and yields shown in Table 4 were obtained by numerical fitting of the experimental data to the proposed models. The equations proved to be consistent in all the cases (Fisher's F ; $\alpha=0.05$; $df=5$), and 94% of the parametric estimates were statistically significant (Student's t ; $\alpha=0.05$; $df=5$), the remaining 6% corresponding to some lag phases. Since this means that the confidence interval for the value of λ includes zero, such a result simply translates the fact that some productions (biomass, lactic acid, pediocin) commence from the start of the culture. Only in one isolated case it reflects an ill-defined production (of acetic acid). On the other hand, the correlation coefficients between observed and expected values were in general very satisfactory (Table 4). Examination of the parametric estimations also showed the following regularities:

Biomass: Peptones from fish wastes led to the highest values for maximum biomass (K in SF is higher 2.2 times than K in MRS-Difco), but the meat (ME, MP) and vegetable (SP) peptones also produced better results than the media usually recommended for LAB culture, among which even a considerable variability of results arose, with differences that reached 50%. The worst protein sources (tryptone and gelatin peptone) rendered biomasses 5.5 and 4 times lower, respectively, than those obtained with SF. Maximum growth rates v_{mX} followed the same trend. The shortest lag phases occurred with shark peptone and pancreatic digest of casein; the longest ones were found with the MRS formulations from Hispanlab and Cultimed.

Lactic acid: Maximum productions did not show significant differences ($\alpha=0.05$) in a wide range of media (S, SP, BP, TR, BP+ME, SF and DIF), were slightly lower in CUL and ME, and 25% lower in tryptone. The highest production rate corresponded to SF peptone, which was higher 1.6 times than the next (meat extract), and higher 3.3 times

than TRY (minimum rate). The lag phases showed considerable differences, with values which were very high (CUL, TR), intermediate (TRY, YT) and low (S, BP, MP, DIF).

Acetic acid: The highest values of A_m and v_{mA} corresponded to SF medium, both surpassing the next (MP) by 50%. Several peptones (shark, skate, gelatine, bactoneopeptone, tryptone, MRS-Oxoid) rendered final values lower than 1 g.L^{-1} and maximum rates lower than $0.025 \text{ g.L}^{-1}.\text{h}^{-1}$, thus showing little ability to promote heterofermentative metabolism. Lag phases showed a wide variability (2.73-28.07 hours), with a high average (~ 16 hours) suggesting that acetic acid, as we shall see, has the kinetic behaviour of a secondary or mixed metabolite. Although Figure 1 shows the fitting to the equation (10) of the values obtained in CUL medium, this case (with a low correlation between observations and predictions) was the only one in which the F -test revealed an inconsistent model; for this reason the parametric values have been excluded from Table 4.

Pediocin: Differences in levels and production rates were particularly notable, from 163 BU/mL and $6 \text{ BU.mL}^{-1}.\text{h}^{-1}$ (gelatine peptone) to over 500 BU/mL and over $40 \text{ BU.mL}^{-1}.\text{h}^{-1}$ (soya and meat extract), there being significant differences even between MRS media from different commercial origin. The results in fish peptones were slightly higher than those obtained in the media located at the lower end of the interval (some MRS being among these).

Yields: Peptones from fish waste provided the highest relationships between biomass production and substrate (carbohydrate or protein) consumption. However –and partly due to the high growth occurring in these cases–, the productivity of these biomasses in lactic acid and pediocin was lower than in the media which promoted low growth rates (TRY, GP and BP).

The variability in the production of pediocin, attributable only to the commercial origin of the MRS medium, had already been detected in previous works [25, 29, 40], and probably translate differences in their peptidic compositions, due to the concrete methods applied in the hydrolysis of the starting protein, or to the type or the condition of the raw material used. Thus, it may be pointed out that the production of pediocin in SF peptone supplemented with yeast extract to the same extent as that of the commercial MRS media

was considerably higher than that previously obtained in a formulation with a lower supplement [25], which demonstrates the role of the cofactors provided by the yeast extract in the biosynthesis of bacteriocin. In tuna peptone, however, the pediocin production was not significantly affected by the presence of yeast extract.

Production of biomass, lactic acid and nisin by L. lactis

A kinetic analysis similar to the preceding one was applied to the nisin-producing species, Lc HD1. Table 5 depicts the parametric estimates and the yields calculated by means of the equations (8, 9, 11, 13-15). All the coefficients were significant (Student's t , $\alpha=0.05$), the models were consistent in all cases (Fisher's F , $\alpha=0.05$), and the correlation coefficients between expected and observed values were very high. The values of these coefficients allowed to establish the following regularities:

Biomass: Production in fish peptones was 2.5 times higher than that obtained in the commercial media, among which among which there were not significant differences. The peptones from chondrichthyes induced the highest growth rates, and the average of the lag phases was, with this microorganism, approximately half that found with *P. acidilactici*.

Lactic acid: Production was very similar in all the media, and only the tuna and shark peptones revealed significantly higher values. Production rates were also higher in the fish peptones, it being in the skate peptone where the highest values of both growth rate and lag phase occurred.

Nisin: Productions and production rates varied considerably, both among the commercial media and those from fish viscera, where the skate and tuna peptones reached an intermediate level (higher than SPP, ME, HIS, and equal to OXO, BP+ME, TRY), and those of swordfish and shark a low level (like MRS-Cultimed).

Yields: As in the case of *P. acidilactici*, and for the same reasons, the media which promoted the highest relationships between biomass production and substrate consumption were those which gave rise to biomasses with the lowest specific productivity of lactic acid and nisin.

Metabolic typification of the bioproductions

Applying the criterion of Luedeking and Piret through equation (12), the estimated values of α and β parameters (Table 6), allowed us to conclude that:

1: With *P. acidilactici*, the lactic acid behaved in all the media as a mixed metabolite, with a low value of the secondary component. The acetic acid displayed a kinetic character which was more dependent on the medium, behaving in the majority of them (60%) as a secondary metabolite, but appearing as a primary one in 7% and as mixed in 33% of the cases. Pediocin followed the kinetics of a mixed metabolite in all the media, with the exception of shark peptone, where it behaved as a primary metabolite (the secondary component was very low in tuna peptone).

2: With *L. lactis*, the lactic acid behaved as a mixed metabolite in 14 of the 16 media, and as primary one in the swordfish and tuna peptones. Nisin –contrarily to pediocin– was a primary metabolite in all the media, with the exception of swordfish and tuna peptones (mixed).

Cluster analysis of the results from different protein sources

Although the yield (production/substrate consumed) is, generally, a more important datum than the efficiency (substrate consumed/initial substrate), when comparing the productions of LAB cultures it is the efficiency, in particular that relative to protein consumption, which presents special interest, due to the complex demands of these microorganisms in peptidic sources. In fact, the media recommended for the culture of LAB always include several peptones at high initial concentrations, of which a very low proportion is consumed. However, when an attempt is made to balance the medium by the usual procedure of reducing the initial level of proteins to a slight excess with regard to the consumption, marked drops in the production of biomass and typical metabolites, in particular bacteriocins, are often found [43].

Moreover, although the studies about the needs of LAB in organic nitrogen sources were not over-conclusive regarding the detection of specific compounds, it has been

demonstrated that amino acids are not essential factors (there is not one amino acid which promotes the response by itself, neither do the joint effects of different amino acids exert significant effects), while it appears important, however, that the medium should contain peptides with 4-20 amino acids, probably including fragments of more or less specific sequences [15, 44-46]. Thus, the medium should provide either precisely the appropriate peptides, or a broader, indeterminate group, of which the microorganism will only use a fraction, but which cannot be reduced without reducing the useful fraction. Consequently, the efficiencies in protein consumptions promoted by different media may be taken as quantitative indications of the degree of adaptation of their peptidic compositions to the needs of the microorganism studied.

In this way, a resource for defining possible improvements in the formulation of media for LAB cultures, aiming to achieve complementarities between peptones of different origins, may consist of the cluster analysis applied to the kinetic values shown in Tables 3 and 4, particularly to the efficiency matrix (Table 7). Said analysis constitutes a well-known tool, applicable to the search of taxonomical relationships [47, 48], likewise to other types of links between microorganisms [49]. In this case, when the Euclidean distances between the results obtained with different protein sources within the two-dimensional space of the bacterial species studied were considered (figure 2), it was revealed that:

1: In any case the meat peptones showed the proximity which might be expected, bearing in mind their common origin from bovine meat. The same absence of similitude was found in the MRS media from different commercial origin (with the only exception of CUL and OXO from the point of view of their efficiencies). The methods of hydrolysis and the condition of the raw material were probably the factors responsible for these differences, as has been stated above.

2: With any of the variables used (efficiency, maximum biomass, growth rate, bacteriocin production rate; lactic acid being the main exception), the peptones from fish residue tend to define small nuclei which were sufficiently remote from the remainder of the peptones to confirm their mutual likeness.

3: While it seems difficult to find a single peptone which maximises the bioproductions of LAB, it seems important, in all cases, to use combinations which include fish peptones, suitable for promoting significantly faster and more massive growth than the majority of the conventional media recommended for the culture of these bacteria.

CONCLUSIONS

Kinetic analysis of *P. acidilactici* and *L. lactis* cultures in a wide spectrum of peptidic sources (both commercial and prepared from fish viscera waste), demonstrated the high variability that the biological, and even commercial origin determines in the bioproductions characteristic of LAB, which are not maximised simultaneously by any of the sources used. The reparametrised models proposed herein allowed the statistically consistent description of the microbial kinetics and the metabolic characterization of the main culture productions. The parameters thus obtained were useful not only for a strict comparison of cultures under different conditions, but also to feed cluster analysis suitable for the identification of the combinations of peptidic sources which promote growth –highly active in fish peptones– and the productions –as primary, secondary or mixed metabolites– associated with the increase in biomass.

Although the cluster analysis is a basically classificatory tool, when it is applied to results of proven descriptive capability it acts as an exploratory method which economizes the subsequent experimentation (especially the factorial experiments to search optimum values), avoiding the selection of superfluous variables and inadequate domains. Fractional or saturated designs (50) also make this work, but their use as an exploratory method is very time-consuming, and they cannot be applied to the results of the indispensable preliminary descriptions –as those concerning to the kinetic profiles of microbial cultures– of the systems under study.

In the context of this work, LAB cultures can have different purposes –as we saw not very compatible–, among them to maximize the growth (as in the preparation of massive starters), or to achieve a certain level of lactic acid, or a certain lactic/acetic ratio (fish silages), or to get a balanced growth of more than one species (as in the preparation of probiotic biomass), or to promote bacteriocin production, and maybe even other. In all

these options, the results of our analysis would allow to define –using the species tested here– a specific peptone combination, as well as a direct and economic experimental plan.

ACKNOWLEDGEMENTS

We wish to thank the Xunta de Galicia (PGIDIT04TAM007001CT) and UE-Life Program (BE-FAIR LIFE05 ENV/E/000267-BE-FAIR) for financial support. Thanks also to Ana Durán and Araceli Menduiña for technical assistance. Dr. José Antonio Vázquez Álvarez had a postdoctoral contract (CSIC-I3P-PC 2003, financed by the European Social Fund). The raw materials were kindly supplied by DILSEA S.L. (Port of Vigo, Spain). The English usage in the manuscript has been revised by J&J Barry Consulting, S.L. (Global Pharmaceutical Support, Vigo, Spain).

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FIGURE CAPTIONS

Figure 1: Kinetics of *P. acidilactici* cultures on the media specified in Table 2. Continuous lines represent the fits of the experimental data (points) to the corresponding mathematical models; discontinuous lines represent the experimental profiles. **RM:** SF (▲), S (●), TR (■), YT (◆); **MRS:** HIS (▲), DIF (●), OXO (■), CUL (◆); **PEPT 1:** BP+ME (▲), TRY (●), BP (■), SPP (◆); **PEPT 2:** PS (▲), PG (●), EC (■), PC (◆). Biomass (X), reducing sugars (RS), lactic acid (L), acetic acid (A), protein-Lowry (Pr), bacteriocin (BT). The corresponding confidence intervals of independent experiments are not shown ($\alpha=0.05$, $n=3$), since these did not transcend in practically any case, the 10% of the experimental mean value.

Figure 2: Cluster analysis of the data relative to maximum values of biomass (K), growth rate (v_{mX}), lactic acid (L_m), lactic acid production rate (v_{mL}), bacteriocin (BT_m), and bacteriocin production rate (v_{mBT}), as well as of the data relative to the protein consumption efficiency matrix (Table 7), expressed as Euclidean distances that separate the media in the two-dimensional space of the lactic acid bacteria.

FIGURES

FIGURE 1

FIGURE 2

TABLES

TABLE 1: Main composition (g/L) of marine peptones (MP) from fish viscera. SF: sword fish; S: shark; TR: thornback ray; YT: yellowfin tuna.

	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
YT	23.6	1.5	4.5

TABLE 2: Composition of the culture media tested (g/L).

	RM ^a	PEPT 1 ^b	PEPT 2 ^c	MRS ^d	MRS ^e	MRS ^f
Glucose	20.00	20.00	20.00	20.00	20.00	20.00
Yeast extract	4.00	4.00	4.00	4.00	5.00	4.00
Sodium acetate	5.00	5.00	5.00	5.00	5.00	5.00
Ammonium citrate	2.00	2.00	2.00	2.00	2.00	2.00
K ₂ HPO ₄	2.00	2.00	2.00	2.00	2.00	2.00
MgSO ₄	0.20	0.20	0.20	0.20	0.10	0.20
MnSO ₄	0.05	0.05	0.05	0.05	0.05	0.05
Tween 80	1.00	1.00	1.00	1.00	1.00	1.00
Meat extract	-	-	-	8.00	-	-
Bactopeptone	-	-	-	10.00	-	10.00
Proteose peptone N°3	-	-	-	-	10.00	-
Beef extract	-	-	-	-	10.00	-
Lab-Lemco powder	-	-	-	-	-	8.00
Marine peptone protein (Lowry)	10.00	-	-	-	-	-
Protein (Lowry) from PEPT 1 ^b	-	10.00	-	-	-	-
Protein (Lowry) from PEPT 2 ^c	-	-	10.00	-	-	-

(^a) RM: Media prepared from marine peptones, as defined in Table 1.

(^b) PEPT 1: Media prepared with commercial peptones TRY, BP, SPP or 10 g/L BP+ 8 g/L ME.

(^c) PEPT 2: Media prepared with commercial peptones SP, GP, ME or MP.

(^d) MRS: Media prepared with commercial formulations HIS or CUL.

(^e) MRS: Medium prepared with commercial formulation from DIF.

(^f) MRS: Medium prepared with commercial formulation from OXO.

BP: Bactoneopeptone (Difco™, Becton, Dickinson and Company, MD, USA).

TRY: Tryptone (Cultimed, Panreac Química S.A., Spain).

SP: Soy Peptone (Cultimed).

SPP: Special Peptone (Oxoid LTD, England).

GP: Gelatine Peptone (Cultimed).

ME: Meat Extract (Difco).

MRS from Hispanlab (HIS), Difco (DIF), Oxoid (OXO) and Cultimed (CUL).

TABLE 3: Symbolic notations used. BU: Bacteriocin arbitrary units.

r_X :	Growth rate. Dimensions: $\text{g.L}^{-1}.\text{h}^{-1}$
X :	Biomass. Dimensions: g/L
t :	Time. Dimensions: h
K :	Maximum biomass. Dimensions: g/L
μ_m :	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. Dimensions: $\text{g.L}^{-1}.\text{h}^{-1}$
λ_X :	Growth lag phase. Dimensions: h
r_L :	Lactic acid rate production. Dimensions: $\text{g.L}^{-1}.\text{h}^{-1}$
L :	Lactic acid. Dimensions: g/L
μ_{mL} :	Specific maximum lactic acid rate production. Dimensions: h^{-1}
L_m :	Maximum lactic acid. Dimensions: g/L
V_{mL} :	Maximum lactic acid rate production. Dimensions: $\text{g.L}^{-1}.\text{h}^{-1}$
λ_L :	Lactic acid lag phase. Dimensions: h
r_A :	Acetic acid rate production. Dimensions: $\text{g.L}^{-1}.\text{h}^{-1}$
A :	Acetic acid. Dimensions: g/L
μ_{mA} :	Specific maximum acetic acid rate production. Dimensions: h^{-1}
A_m :	Maximum acetic acid. Dimensions: g/L
V_{mA} :	Maximum acetic acid rate production. Dimensions: $\text{g.L}^{-1}.\text{h}^{-1}$
λ_A :	Acetic acid lag phase. Dimensions: h
r_{BT} :	Bacteriocin (pediocin or nisin) rate production. Dimensions: $\text{BU.mL}^{-1}.\text{h}^{-1}$
BT :	Bacteriocin (pediocin or nisin). Dimensions: BU/mL
μ_{mBT} :	Specific maximum bacteriocin rate production. Dimensions: h^{-1}
BT_m :	Maximum bacteriocin. Dimensions: BU/mL
V_{mBT} :	Maximum bacteriocin rate production. Dimensions: $\text{BU.mL}^{-1}.\text{h}^{-1}$
λ_{BT} :	Bacteriocin lag phase. Dimensions: h
r_P :	Production rate for product (X, L, A, BT). Dimensions: $\text{g.L}^{-1}.\text{h}^{-1}$ or $\text{BU.mL}^{-1}.\text{h}^{-1}$
α :	Luedeking and Piret parameter (growth-associated constant for product formation). Dimensions: $\text{g product / g biomass}$ or BU/mg
β :	Luedeking and Piret parameter (non growth-associated constant for product formation). Dimensions: $\text{g product.g}^{-1}(\text{biomass}).\text{h}^{-1}$ or $\text{BU.mg}^{-1}.\text{h}^{-1}$
P :	Product concentration (X, L, A, BT) formed by the microorganism. Dimensions: g/L or BU/mL
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: $\text{g product or BU / g protein}$
$Y_{P/RS}$:	Product formation / reducing sugars consumption. Dimensions: $\text{g product or BU / g reducing sugars}$
$Y_{BT/X}$:	Bacteriocin production / biomass production. Dimensions: BU / g biomass

TABLE 4: Parametric estimations (see Table 3) corresponding to the kinetic models (8, 9-11, 13-15), applied to cultures of *P. acidilactici* on specified media (see Table 2). Intervals of correlation coefficients between observed and expected productions were: $r(X)=0.988-0.999$; $r(L)=0.989-0.999$; $r(A)=0.935-0.999$; $r(BT)=0.984-0.999$.

MEDIA		BIOMASS (X)			LACTIC (L)			ACETIC (A)			BACTERIOCIN (BT)			YIELDS (Y)								
		K	V_{mX}	λ_X	L_m	V_{mL}	λ_L	A_m	V_{mA}	λ_A	BT_m	V_{mBT}	λ_{BT}	$Y_{X/Pr}$	$Y_{X/RS}$	$Y_{L/Pr}$	$Y_{L/RS}$	$Y_{A/Pr}$	$Y_{A/RS}$	$Y_{BT/Pr}$	$Y_{BT/RS}$	$Y_{BT/X}$
RM	SF	2.898	0.157	4.150	9.636	0.709	7.046	3.277	0.098	18.762	258.09	16.583	7.258	1.413	0.158	4.590	0.512	1.415	0.158	123259	13753	87220
	S	2.308	0.079	3.475	10.138	0.305	3.267	0.558	0.023	2.733	242.11	16.667	7.278	1.505	0.159	6.697	0.708	0.368	0.039	158606	16758	105375
	TR	2.037	0.084	7.734	9.875	0.377	10.089	0.542	0.022	6.680	301.57	13.489	14.212	1.187	0.152	5.983	0.767	0.282	0.036	172282	22074	145167
	YT	1.485	0.077	6.054	9.125	0.433	6.942	1.390	0.038	23.372	265.65	13.880	9.128	1.284	0.099	8.190	0.630	0.940	0.072	228853	17616	178287
MRS	HIS	1.167	0.064	9.339	7.973	0.270	9.387	1.171	0.037	18.184	241.49	16.861	17.053	1.044	0.103	6.812	0.670	0.923	0.091	213718	21030	204624
	DIF	1.317	0.090	6.658	9.827	0.388	3.907	1.619	0.055	18.985	494.58	65.104	11.868	1.238	0.091	9.214	0.675	1.482	0.108	445054	32579	359380
	OXO	0.871	0.071	5.563	8.925	0.443	4.721	0.675	0.022	14.024	268.43	33.339	11.138	0.971	0.068	10.176	0.710	0.648	0.045	276923	19310	285068
	CUL	1.073	0.054	8.480	9.454	0.287	9.763	-	-	-	237.54	34.648	17.441	1.186	0.089	9.624	0.725	0.656	0.049	281570	21203	237407
PEPT 1	BP+ME	1.282	0.109	6.538	9.707	0.353	3.417	0.997	0.025	13.681	406.54	31.357	8.771	1.163	0.106	9.216	0.841	0.874	0.080	381459	34821	327978
	TRY	0.529	0.023	2.435	7.130	0.217	6.895	0.813	0.024	28.074	221.65	7.460	14.481	0.838	0.055	11.123	0.733	0.923	0.061	304769	20071	363486
	BP	0.888	0.084	5.307	9.921	0.296	3.390	0.596	0.012	14.505	210.19	13.488	5.454	1.020	0.078	11.022	0.844	0.505	0.039	247868	18971	243060
	SPP	1.102	0.077	7.162	8.686	0.311	6.356	0.906	0.066	18.912	445.76	37.135	13.584	1.012	0.076	8.248	0.618	0.862	0.065	424229	31803	419229
PEPT 2	SP	1.467	0.099	7.345	10.017	0.413	5.311	1.439	0.064	19.098	545.92	43.599	12.389	1.423	0.090	10.058	0.639	1.577	0.100	505846	32137	355459
	GP	0.715	0.044	4.912	8.126	0.268	6.222	0.401	0.021	18.707	163.47	6.044	8.055	1.319	0.063	15.574	0.743	0.926	0.044	301556	14385	228708
	ME	1.651	0.117	7.321	9.381	0.446	5.751	1.644	0.053	15.586	516.13	58.231	11.939	0.904	0.106	5.348	0.624	0.824	0.096	269182	31421	297676
	MP	1.391	0.077	6.772	8.101	0.310	3.475	2.061	0.065	15.137	442.29	35.838	12.847	1.003	0.073	5.943	0.432	1.383	0.101	327943	23835	327016

TABLE 5: Parametric estimations (see Table 3) corresponding to the kinetic models (8, 9-11, 13-15), applied to cultures of *L. lactis* on specified media (see Table 2). Intervals of correlation coefficients between observed and expected productions were: $r(X)=0.996-0.999$; $r(L)=0.994-0.999$; $r(BT)=0.989-0.999$.

MEDIA		BIOMASS (X)			LACTIC (L)			BACTERIOCIN (BT)			YIELDS (Y)						
		K	V_{mX}	λ_X	L_m	V_{mL}	λ_L	BT_m	V_{mBT}	λ_{BT}	$Y_{X/Pr}$	$Y_{X/RS}$	$Y_{L/Pr}$	$Y_{L/RS}$	$Y_{BT/Pr}$	$Y_{BT/RS}$	$Y_{BT/X}$
RM	SF	1.711	0.163	2.134	6.000	0.643	2.701	8.428	1.252	5.291	1.141	0.209	4.301	0.787	5685	1040	4982
	S	1.947	0.294	3.283	6.974	0.978	3.245	34.050	5.289	1.820	1.138	0.194	4.365	0.743	19159	3260	16832
	TR	2.633	0.454	3.604	6.221	1.702	5.243	11.959	2.020	5.189	1.221	0.257	3.179	0.669	5443	1146	4459
	YT	1.606	0.168	2.874	7.769	1.053	3.392	32.036	4.112	2.855	1.077	0.138	5.295	0.680	21356	2745	19835
MRS	HIS	1.027	0.217	3.366	6.358	0.901	3.142	26.319	4.059	2.277	0.874	0.116	6.036	0.803	22732	2745	26006
	DIF	0.976	0.191	3.053	6.850	0.865	2.288	43.352	10.433	2.885	0.676	0.115	5.213	0.887	30338	3024	44848
	OXO	0.917	0.169	2.790	6.409	0.742	1.773	36.866	8.750	2.202	0.825	0.135	5.991	0.984	31645	5164	38379
	CUL	0.952	0.253	4.008	6.356	0.835	3.325	17.319	2.987	2.030	0.902	0.115	6.539	0.837	16216	5196	17978
PEPT 1	BP+ME	0.970	0.171	3.423	6.301	0.824	3.299	34.899	5.821	2.031	0.890	0.134	5.963	0.895	33991	5101	38204
	TRY	0.944	0.189	3.598	6.336	0.663	2.687	33.294	4.433	1.784	0.828	0.117	5.670	0.804	28235	4004	34107
	BP	0.928	0.193	3.032	6.440	0.767	3.063	50.798	9.404	2.239	0.661	0.111	4.904	0.823	37556	6306	56839
	SPP	0.935	0.218	3.603	6.418	0.765	3.119	27.658	5.060	2.164	0.842	0.123	6.229	0.908	25468	3711	30240
PEPT 2	SP	0.952	0.205	3.712	6.497	0.834	3.172	48.074	6.846	2.444	0.663	0.110	4.878	0.808	33928	5621	51150
	GP	0.958	0.161	2.728	6.427	0.702	3.416	49.313	9.864	3.066	0.681	0.095	4.883	0.682	34234	4781	50268
	ME	0.985	0.253	4.395	6.297	0.760	3.213	29.040	6.209	2.390	0.768	0.119	5.496	0.854	24336	3781	31685
	MP	0.934	0.204	3.858	6.665	0.679	2.459	38.652	7.947	1.947	0.823	0.136	6.264	1.033	34709	5724	42188

TABLE 6: Parametric estimations of the Luedeking-Piret equation (12) applied to the productions of lactic acid, acetic acid and bacteriocins (pediocin and nisin). Intervals of correlation coefficients between observed and expected values were, for Pc 1.02: $r(\text{lactic})=0.988-0.999$; $r(\text{acetic})=0.931-0.996$; $r(\text{pediocin})=0.957-0.993$; for Lc HD1: $r(\text{lactic})=0.991-0.999$; $r(\text{nisin})=0.944-0.998$.

		<i>Pediococcus acidilactici</i> (Pc 1.02)						<i>Lactococcus lactis</i> (Lc HD1)			
MEDIUM		LACTIC		ACETIC		PEDIOCIN		LACTIC		NISIN	
		α	β	α	β	α	β	α	β	α	β
RM	SF	19.463	0.075	-	0.222	477.35	3.469	9.915	-	10.842	0.307
	S	23.579	0.385	2.028	-	867.14	-	9.060	0.062	50.169	-
	TR	19.951	0.699	1.645	0.017	428.72	28.661	6.428	0.071	8.867	0.311
	YT	28.814	0.522	-	0.168	761.59	18.146	14.357	-	57.740	-
MRS	HIS	22.704	1.015	0.380	0.243	511.60	42.052	13.526	0.349	71.772	-
	DIF	28.780	0.690	-	0.267	1729.3	24.533	16.468	0.271	129.46	-
	OXO	37.631	0.876	-	0.162	1299.0	17.838	16.415	0.257	118.96	-
	CUL	25.066	1.419	-	-	926.14	31.385	13.203	0.504	52.701	-
PEPT 1	BP+ME	22.460	0.851	-	0.151	1257.5	23.114	14.456	0.276	102.71	-
	TRY	30.126	2.180	-	0.222	46.933	95.642	12.518	0.503	97.324	-
	BP	20.962	1.556	-	0.098	937.36	16.437	12.385	0.596	157.88	-
	SPP	21.798	1.074	0.369	0.199	1157.1	57.246	12.524	0.582	85.728	-
PEPT 2	SP	26.631	0.660	-	0.244	1388.9	38.897	14.232	0.417	138.15	-
	GP	25.172	1.720	-	0.135	510.07	34.444	10.326	0.691	146.60	-
	ME	23.358	0.490	0.031	0.230	1486.2	16.612	12.633	0.454	85.792	-
	MP	23.457	0.295	0.438	0.342	1241.4	37.297	13.463	0.254	120.98	-

TABLE 7: Matrix of efficiencies in protein consumptions (initial level $\times$ 100 / final level) for all the combinations of media and lactic acid bacteria

	SF	S	TR	YT	HIS	DIF	OXO	CUL	B+M	TRY	BP	SPP	SP	GP	ME	MP
Pc	82.6	87.6	86.0	90.6	90.0	91.3	91.7	91.6	91.4	94.9	92.9	91.4	91.6	95.8	85.5	88.9
Lc	88.6	86.7	83.3	88.4	90.6	89.3	90.6	90.8	91.7	90.9	89.6	91.4	90.0	89.3	90.1	91.0