Modelling and validation of *Lactobacillus plantarum* fermentations in cereal-based media with different sugar concentrations and buffering capacities

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Abstract

An unstructured mathematical model is proposed to describe the fermentation kinetics of growth, lactic acid production, *pH* and sugar consumption by *Lactobacillus plantarum* as a function of the buffering capacity and initial glucose concentration of the culture media. Initially the experimental data of *L. plantarum* fermentations in synthetic media with different buffering capacity and glucose were fitted to a set of primary models. Later the parameters obtained from these models were used to establish mathematical relationships with the independent variables tested. The models were validated with 6 fermentations of *L. plantarum* in different cereal-based media. In most cases the propose models adequately describe the biochemical changes taking place during fermentation. These proposed equations and studied effects are promising approach for the optimization and formulation of cereal-based functional foods.

Keywords *Lactobacillus plantarum*; probiotic; mathematical models; cereal-based media; buffering capacity; sugar concentration.

Introduction

The mathematical models that are used to simulate a bioprocess can generally be classified as unstructured or structured. In unstructured models the biomass is considered as one entity described only by its concentration. These models do not take into account any changes that could take place in the inner cells. In structured models the biomass is defined and includes intracellular components, such as the RNA content, enzymes, reactants and products [1]. Although the structured models provide a better understanding of the modelled system, unstructured models are mainly used to describe bacterial kinetics in complex natural substrates. This is mainly due to the complexity of the substrates and to the difficulties in obtaining large sets of experimental data for the intracellular components [2].

In food microbiology, mathematical modelling has been mainly applied to predict growth or inactivation of spoilage of bacteria and foodborne pathogens [3-6]. A number of models have been used to describe the sigmoidal curves of bacterial growth, such as the model of Gompertz [7], Richards [8], Stannard et al. [9], Schnute [10], the logistic model and others. These equations can fit cell growth over time and take into account growth inhibition in the stationary phase of growth.

In the last ten years, there has been an increasing interest in modelling the kinetics of beneficial microorganisms in food systems. For lactic acid bacteria (LAB) the models used to describe cell growth are the unstructured and structured equations mentioned earlier. Growth or non-growth related models are also applied to describe the changes of other biochemical compounds and physical properties in these food systems. These changes include primary or secondary

metabolites concentrations, volatile production as well as rheological and textural properties [11-14]. The aim of these models is to mathematically relate the biochemical properties (response variables) to environmental factors (controlling factors), such as temperature, *pH*, water activity and substrate composition. This contributes to a better understanding and control of the fermentation process and helps to clarify how and to what extent the environment will interfere with the behaviour of the strains [11].

In general modelling is performed in two stages. In the first stage the primary models are applied to the experimental data describing the change of a response variable over time. In the second stage secondary models are developed expressing the bio-kinetic parameters derived from the primary models as a function of a single environmental factor. It must be noted that both primary and secondary models are built using data from experiments in synthetic media under carefully controlled conditions. The predictability of the models is then assessed in the complex food systems. LAB research has focused so far in modelling the dependence of the growth rate on temperature and *pH* at *pH*-controlled conditions [15-17]. Very little research has been done in the secondary modelling of growth when *pH* is not controlled, or taking into account other bio-kinetic parameters, such lactic acid and bacteriocins production [18-22].

On the other hand, cereals are one of the most suitable components for the production of foods contains a probiotic microorganism (in most cases lactic acid bacteria or bifidobacteria) and a prebiotic substrate. Traditionally, probiotic microorganisms have been included in dairy products, meat and fruit juices. The definition and development of new functional foods cereal-based combine the beneficial effects of cereals and health promoting bacteria is a challenging issue [23,24]. In this sense, the major biochemical properties affecting the functionality and quality of a

probiotic-containing food product are the cell population, lactic acid concentration and *pH*. The cell concentration in the end product is an indicator of probiotic functionality, the lactic acid influences the organoleptic properties and also acts as a preservative agent, while the *pH* is the main factor determining the stability and safety of the product during storage [25,26]. Since sugar is the main carbon source used by the cells, knowledge of the sugar kinetics contributes to a better understanding of cell growth and product formation. Moreover, the amount of sugar present in the carrier medium influences the survival of *L. plantarum* in acidified model solutions imitating stomach conditions.

The aim of this study was to develop a model that would be able to simulate the kinetics of cell growth, lactic acid production, *pH* drop and sugar consumption in cereal-based fermentations with *L. plantarum*. The kinetic parameters of the primary models for these dependent variables were expressed as a function of the initial sugar concentration of the media and their buffering capacity. The models were built using data from fermentations in synthetic media with various levels of glucose and different buffering abilities. Finally, the predictability of the models was evaluated using a variety of cereal-based fermentations of different concentrations. Thus, the numerical parameters obtained allowed the characterisation of these cultures and could be a preliminary step in the formulation of novel potentially probiotic foods.

Materials and methods

Microorganism and inoculum

The strain *L. plantarum* NCIMB 8826 isolated from human saliva was used. It was maintained at 4ºC and sub-cultured monthly on MRS agar slopes (Oxoid). Isolated colonies from MRS agar

plates were pre-cultured twice in MRS broth (Oxoid) for approximately 24 h at 37ºC. The cells were collected by centrifugation (5000 g, 10 min, 4ºC), washed twice with sterile quarter-strength Ringer's solution and re-suspended in the same solution. The bacterial suspensions used as inocula for the fermentation studies (1% v/v) were obtained from 12 h pre-cultured cells.

Culture media and microbiological methods

The synthetic media used for the studies of the buffering capacity and the effect of sugars on *L. plantarum* growth are summarized in Table 1. The *pH* of all media was adjusted to about 5.8 with 1N HCI or 1N NaOH and the media sterilised for 10 min at 121ºC. Six cereal media were used for model validation: 20% wheat extract, 30% barley extract, 40% barley extract, 30% barley extract supplemented with 2.2 g l^{-1} citric acid, and 5% and 20% malt extract. All cereal-based media were prepared as described in Charalampopoulos et al. [27]. In all cases the *pH* was initially adjusted to about 6.0 with 1N HCI or 1N NaOH and the media sterilised for 30 min at 121ºC.

Shake-flask fermentations were performed in triplicate using 500 mL screw-capped glass bottles without oxygen control. Bottles were inoculated with a 1% v/v of lactic acid bacteria and incubated at 150 rpm and 37°C for 30 or 42 h. The viable cell counting method was used for cell enumeration [28]. Cell growth was monitored by measuring the optical density of the media at 600 nm. The optical density values were transformed to cell counts ($\log_{10}$ cfu ml^{-1}) using a pre-established calibration curve. In the fermentation samples *pH*, reducing sugar (as glucose) and lactic acid content were analysed.

Buffering capacity

The buffering agent used was a 0.2 M acetate buffer stock solution, from which 8 media of different buffering capacity were prepared by dilution (from 1/1 to 1/15). The buffering capacity of the media was measured after addition of the nutrient substrate using the method of Pai et al. [29]. 100 ml of each medium were titrated with 1N HCl. The values were expressed as the amount of HCl (mmoles) required to drop one *pH* unit per unit volume (1 litre).

Analytical methods

The dinitrosalicylic acid (DNS) assay was used to measure the reducing sugar concentration in the supernatants of the fermented cereal extracts according to Miller [30]. A standard curve was made using glucose at various concentrations. Lactic acid was measured using an enzymatic kit for D- and L- lactic acid (Boehringer Mannheim).

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 Mathematica 6 (Wolfram Research, Inc.) programs were used to evaluate the significance of the parameters estimated by fitting of the experimental values to the proposed mathematical models, and the consistency of these equations.

Results and Discussion

A set of four non-linear algebraic equations comprising the primary models was used to describe

cell growth (*N*), lactic acid production (*P*), *pH* and sugar concentration (*S*) with time. The model parameters were estimated from the 17 batch data of *N*, *P*, *pH* and *S vs*. time. The parameters of the primary models were then fitted to the controlling factors (secondary models) by using linear or non-linear regression analysis. The definition and units of the model parameters and variables are shown in Table 2.

Growth models

A common model to describe cell population growth is the differential equation proposed by Velhurst in 1844, which includes an inhibition factor of growth. By assuming that inhibition of a population *N* is proportional to N^2, the growth rate is given by the following equation

$$\frac{dN}{dt} = \mu_m N\left(\frac{K-N}{K}\right) \tag{1}$$

Integrating between $N_0 \rightarrow N$ and $0 \rightarrow t$ gives the biomass concentration as a function of time [31,32]

$$N = \frac{K}{1+\exp\left(c-\mu_m t\right)} \text{ , with } c = \ln\left(\frac{K}{X_0}-1\right) \tag{2}$$

In order to give biological meaning to the parameters when $\log_{10}N$ are used, equation 2 was reparameterised according to Zwietering et al. [33]

$$X = \log\left(\frac{N}{N_0}\right) = \frac{A}{1+\exp\left[2+\frac{4\mu_m}{A}(\lambda - t)\right]} \quad (3)$$

The data of the 17 batch cultures were fitted using equation 3. The optimum parameters with the 95% confidence intervals and consistence of the equation (Fisher's *F* test, α=0.05) are presented in Table 3. In all cases, the fit of results was statistically satisfactory. The mathematical equations were consistent (Fisher's *F* test) and the parametric estimations were significant (Student's t test). The values predicted by equation 3 are highly correlated with the experimental data with a regression coefficient r > 0.97.

These results indicate that the logistic model could be used to describe *L. plantarum* growth in various environmental conditions. This means that the inhibitory effect of the accumulated cell concentration on μ_m (as assumed by equation 1) can explain various types of growth limitations such as low sugar availability. It could also explain the inhibitory effect of *pH*, which illustrated by the fact that μ_m decreases with buffering capacity. Lejeune et al. [34] justify this by pointing out that the cell concentration depends on the amount of nutrients consumed and the lactic acid produced (and consequently the *pH*). For these reasons the logistic model, or similar forms of it, have been widely used to model the growth of LAB [35-36].

Since the aim of this study was to create a link between cell growth and the properties of the starting media, the bio-kinetic parameters μ_m, *A* and λ were expressed as a function of buffering capacity and glucose. All the functions were of the form $p(f) = p_{opt} \times \gamma(f)$, which indicates that an optimum value of the parameter (p_{opt}) is obtained when the controlling factor (f) is also optimum.

The function $\gamma(f)$ describes the response of the growth to changes in the factor f, with values of f between 0 (no response) and 1 (optimum response). By assuming that the influence of a factor is independent of other factors, the model describing the combined effects of the factors f_1, f_2, f_3,….,f_n would then be $p(f_1, f_2, f_3,\ldots,f_n) = p_{opt} \times \gamma(f_1, f_2, f_3,\ldots,f_n)$. This concept is generally called the γ-concept and has been extensively used to relate the bio-kinetic parameters describing cell growth (and in fewer cases primary or secondary metabolic activities) to the environmental conditions (usually *pH* or temperature) [11,16,37-40].

Secondary modelling of μ_m

Figure 1 (up) shows the effect of glucose (S) on μ_m. The data show that μ_m increases with the glucose concentration from 0 to 6 g l^{-1}, while between 6 and 20 g l^{-1} μ_m remains approximately constant. The hyperbolic shape of the trend was described using the Monod model [41]:

$$\mu_m = \mu_{optS} \frac{S}{K_s + S} \qquad (4)$$

The fit of equation 4 to the μ_m values (obtained from Table 3) was performed by the non-linear least squares method. The fit was satisfactory and the derived parameters were 0.70 h^{-1} for μ_{optS} and 1.5 g l^{-1} for K_s (see Table 4 for statistical validation)

The small K_s value suggests that the sugar requirement of this strain is relatively low, and the strain could be used to ferment media of low sugar content. Venkatesh et al. [42] in *pH*-controlled media monitored growth of *L. bulgaricus* and showed that K_s is *pH* dependent. In the

present study the buffering capacities of the 8 batches were the same (1.09 mmoles HCl pH^{-1} l^{-1}). The *pH* effect on K_s was probably not significant. Burgos-Rubio et al. [43] reported a K_s value of 3.36 g l^{-1} for *L. bulgaricus*, while Martens et al. [38] did not observe any dependence of *L. plantarum* μ_{optS} on the initial sugar concentration of the medium. For this reason they considered a K_s value of 0.072 g l^{-1}, which is the general value for *E. coli* on glucose [44]. It must be pointed out that little information is available in the literature regarding the kinetics of LAB growth in a fermented food product. Most published works study the kinetics of the fermentation process for the optimization of the production of lactic acid in industrial applications. In these cases fermentations are carried out under *pH*-control using media with very high sugar concentrations (60 to 300 g l^{-1}). This is the reason why K_s is considered to be very small and usually is neglected.

It can be also observed from the data presented in Table 3 that μ_m increases with the buffering capacity of the medium. This can be attributed to the fact that in media with low buffering capacity the *pH* drop was greater. The inhibitory effect of *pH* takes place earlier in the fermentation process, resulting in lower μ_m values. A linear relationship was observed between μ_m and the buffering capacity, and the μ_m value corresponding to the higher buffering capacity (1.09 mmoles HCl pH^{-1} l^{-1}) was considered to be the optimum value (μ_{optB} = 0.61 h^{-1}) for these working conditions. The relative μ_m values (μ_m / μ_{optB}) were then plotted against the buffering capacity (B) in order to obtain an equation of the form $\mu_m = \mu_{optB} \times \gamma$ (Figure 1, down). As B can not be zero the data were centered on the mean values of B ($B_{cent} = B - B_{average}$; $B_{average}$ = 0.57 mmoles pH^{-1} l^{-1}) [31]. The following linear equation was then used to fit the data

$$\frac{\mu_m}{\mu_{optB}} = \alpha_1 + \beta_1 \left(B - 0.57 \right) \quad (5)$$

The fit was satisfactory (r = 0.98, *F*-value= 623.04, Table 4), which suggests that equation 5 can appropriately describe the dependency of μ_m with the buffering capacity.

By assuming that the influences of sugar and buffering capacity on μ_m are independent, equations 4 and 5 could be combined

$$\mu_m = \mu_{opt} \left(\frac{S}{K_s + S} \right) \left[\alpha_1 + \beta_1 \left(B - 0.57 \right) \right] \quad (6)$$

where μ_{opt} (h^{-1}) is the μ_m value at the conditions where both controlling factors are at their optimum, which occurs when *B* is 1.09 mmoles HCl pH^{-1} l^{-1} and *S* is much greater than K_s. It must be noted that according to equation 6, when *S* = 20 g l^{-1} and *B* = 1.09 mmoles HCl pH^{-1} l^{-1} the value of μ_{opt} would be 0.64 h^{-1}, which is slightly higher than the μ_{optB} value of 0.61 h^{-1} calculated experimentally. Of the three possible μ_{opt} values (μ_{opt} obtained from equation 6, μ_{optS} and μ_{optB}), the one with the smaller coefficient of variation is μ_{optS}, and this value was the one considered in the equation describing the joint effect (equation 6)

$$\mu_m = 0.70 \left(\frac{S}{1.40 + S} \right) \left[0.54 + 0.75 \left(B - 0.57 \right) \right] \quad (7)$$

Secondary modelling of A

Figure 2 (up) illustrates the dependence of the maximum relative cell concentration (*A*) with the glucose concentration (*S*). It can be observed that the *A* values increase with the initial sugar concentration of the medium until a stationary value is reached. This behaviour was similar to μ_m and therefore a Monod type equation was used to describe the dependence of *A* with the sugar concentration (replacing μ_{optS} by A_{optS} and K_S by $K_S^{'}$).

The fit of the experimental data to this equation was satisfactory although the estimation of $K_S^{'}$ has a considerable error (see statistical parameters in Table 4). This could explain the observed difference between $K_S^{'}$ and K_S (2.52 and 1.40 g l^{-1} respectively), although it is also possible that in the media containing small amounts of glucose, the efficiency of glucose consumption for cell conversion decreases. A similar behaviour has been reported by Schepers et al. [40] in *L. helveticus*.

In the batches where the buffering capacity was the controlling factor, the *A* values ranged from 2.12 to 3.11 [$\log_{10}(N/N_0)_{max}$], suggesting that the effect of buffering capacity was less significant than that of glucose. This means that even in media where the *pH* drops fast, the final cell concentration would reach a relatively high level providing that there is sufficient carbon source. Since *A* continuously increases with increasing buffering capacity, although in a non-linear manner, the value of *A* corresponding to the highest buffering capacity (1.09 mmoles HCl pH^{-1} l^{-1}) was considered to be the optimum value (A_{optB} = 3.11). The relative *A* values (A/A_{optB}) were then plotted (figure 2, down) *vs*. the buffering capacity (*B*). The data were also centered on the mean value of the *B*. The following 2nd order polynomial equation was then used to fit the data

$$A = 3.89\left(\frac{S}{2.52+S}\right)\left[0.95+0.39\left(B-0.57\right)-0.64\left(B-0.57\right)^2\right] \quad (8)$$

Secondary modelling of λ

In order to describe the dependency of the lag time (λ) on environmental factors, such as temperature and *pH*, other researchers have modelled λ in the same way as the inverse of μ_m [15,16,38], and this strategy was also used in this work. As shown in figure 3, a strong linear correlation existed between $\mu_m \lambda$ and μ_m (r = 0.99, *F*-value = 1768.4), which could be described by the relationship

$$\mu_m \lambda = \alpha_3 + \beta_3 \mu_m \quad (9)$$

The values of the regression parameters are α_3 = -0.26 ± 0.14 and β_3 = (4.17 ± 0.32) h. These parameters were obtained from the whole set of batches and are very similar to the ones that would be obtained from the batches with a constant substrate concentration and buffering capacity separately. Equation 9 can then be used to predict the lag phase of *L. plantarum* by introducing the value of μ_m obtained from equation 7.

Lactic acid production models

Homofermentative LAB meet their energy requirements for growth and maintenance by producing lactic acid. Maintenance is the energy required for survival or for preservation of cell viability, which is not directly coupled with the synthesis of new cells [45]. The uncoupled lactic acid production by *L. plantarum* was clearly observed in these batches when lactic acid

production continued after growth had ceased (data not shown). Based on this, a kinetic representation for lactic acid production could the classical Luedeking-Piret model [46]. This model suggests that the product (*P*) formation rate depends on the growth rate (*dN/dt*) and the cell concentration (*N*). We have expressed the cell concentration in terms of the relative cell population (*X*)

$$\frac{dP}{dt} = \alpha_p \frac{dX}{dt} + \beta_p X \tag{10}$$

Integration of equation 10 between $P_0 \rightarrow P$, $0 \rightarrow X$ and $0 \rightarrow t$ gives

$$P = P_0 + \frac{\alpha_p A}{1+\exp\left[2+\frac{4\mu_m}{A}(\lambda - t)\right]} + \frac{\beta_p A^2}{4\mu_m} \cdot \ln\left[\frac{\exp\left(2+\frac{4\mu_m \lambda}{A}\right)+\exp\left(\frac{4\mu_m t}{A}\right)}{1+\exp\left(2+\frac{4\mu_m \lambda}{A}\right)}\right] \tag{11}$$

In Table 5 the parameters of this equation with their confidence intervals (α = 0.05), the *F*-value and correlation coefficients are summarized.

Secondary modelling of parameter α_p

The values for the growth associated constant α_p varies between 0.64 and 0.84 g lactic acid $(\log_{10}(N/N_0))^{-1}$ l^{-1} in the batches where buffering capacity was the controlling factor (Table 5). When α_p was plotted against the buffering capacity no clear tendency was observed and the straight line fitting the data was more or less horizontal. For this reason α_p was assumed to be constant in our range of buffering capacity. The values of α_p in the batches where sugar was the controlling

factor varied between 0.42 and 0.82 g lactic acid $(\log_{10}(N/N_0))^{-1}\ l^{-1}$ (Table 5). In this case α_p increases with the sugar concentration. Fitting α_p *vs*. *S* data with a 2nd order polynomial equation was not appropriate (r = 0.75) but the fit considerably improved by including 3rd or 4th order terms. However, this type of model would be unrealistic and difficult to interpret [47] and for that reason the mean α_p value in all batches was used to describe the growth associated lactic acid production ($\alpha_{p\text{mean}} = 0.71 \pm 0.04$ g lactic acid $(\log_{10}(N/N_0))^{-1}\ l^{-1}$).

Secondary modelling of parameter β_p

In the batches where the buffering capacity was the controlling factor, when β_p was plotted against *B* not a clear trend was observed and β_p was considered to be independent of the buffering capacity. The values of β_p when sugar was the controlling factor ranged from 0 to 0.021 g lactic acid $(\log_{10}(N/N_0))^{-1}\ l^{-1}\ h^{-1}$ (Table 5). In one of the batches (when 4 g l^{-1} of sugar concentration was used) β_p was negative and statistically not significant. The significant values of β_p were then plotted as a function of sugar (*S*). A lineal equation provide a satisfactory fit of these data (r = 0.964, *F*-value = 114.1) with parametric estimations α_4 = 0.0007 (not significant) and $\beta_4 = 0.0011 \pm 0.0003$. The equation that describes β_p as a function of the sugar concentration will then be

$$\beta_p = 0.0011S \qquad (12)$$

pH model

Several authors have proposed different approaches to know the dependence between *pH* and fermentation parameters such as enzyme reaction rates [48], specific growth rates and specific

activities [49] or by means of re-alkalized fed-batch cultures [50]. In order to model the evolution of *pH* during fermentation we assumed that the *pH* drop during growth of *L. plantarum* was exclusively due to lactic acid production. Thus, with the purpose to establish a relationship between *pH* and lactic acid production (*P*) the *pH* evolution in media with different buffering capacity was studied. In the initial stages of the exponential phase of growth the *pH* decreased faster in the media with lower buffering capacity, and the final *pH* value was always lower in media with lower buffering capacity. A mechanistic model [31] was then used assuming that the *pH* drop with respect to the lactic acid production is directly proportional to the *pH* minus the final *pH* ($pH_{\min}$)

$$\frac{dpH}{dP} = k\left(pH - pH_{\min}\right) \tag{13}$$

integration of equation 13 with initial conditions P_0 and pH_0 yields

$$pH = pH_{\min} + \left(pH_0 - pH_{\min}\right)e^{-k(P-P_0)} \tag{14}$$

The values of these parameters were estimated by fitting equation 14 to the experimental data for lactic acid and *pH* (in the batches with different buffering capacities) using the non-linear least squares method. The $pH_{\min}$ values did not show any obvious dependency on the buffering capacity (*B*), and the mean value 3.21±0.06 was used to express the $pH_{\min}$ in all cases. The *k* values decrease with increasing buffering capacity, which is illustrated in figure 4. This trend is adequately described by a 2nd order polynomial equation (r = 0.997, *F*-value = 865.1) and *k* can be expressed by

$$k = 5.81 - 9.95B + 4.53B^2 \quad (15)$$

Finally, a full model of *pH* can be obtained by introducing equation 14 in equation 11.

$$pH = pH_{\min} + \left(pH_0 - pH_{\min}\right)\exp\left\{-k\left[\frac{\alpha_p A}{1+e^{\left[2+\frac{4\mu_m}{A}(\lambda-t)\right]}} + \frac{\beta_p A^2}{4\mu_m}\cdot\ln\left[\frac{e^{\left[2+\frac{4\mu_m}{A}(\lambda-t)\right]}+e^{\left(\frac{4\mu_m t}{A}\right)}}{1+e^{\left[2+\frac{4\mu_m}{A}(\lambda-t)\right]}}\right]\right]\right\} \quad (16)$$

Sugar consumption model

The rate of sugar consumption is mainly a function of three factors: the growth rate, lactic acid production and the rate of substrate uptake for cell maintenance. These three factors can be put together using yields and maintenance coefficients according to the following equation [51]

$$\frac{dS}{dt} = -\frac{1}{Y_{p/s}}\frac{dP}{dt} - \frac{1}{Y_{x/s}}\frac{dX}{dt} - m_s X \quad (17)$$

Introducing the logistic growth model 3 and the lactic acid production model 11 into equation 17 and integrating, a model describing the change of sugar concentration with time could be obtained. This equation was used to fit the experimental data using the non-linear least squares in order to estimate the parameters $Y_{p/s}$, $Y_{x/s}$ and m_s. Though the iterative process converged to a final solution, the parameters obtained were not realistic (and not significant) and depended on the initial values of the estimated parameter. Similar problems in the estimation of yields and maintenance coefficients have been reported before [35,52]. Esener et al. [53] also suggested that

the estimation of the maintenance coefficients from batch fermentation data has a considerable error.

Based on this it was assumed that the amount of sugar used for cell growth and maintenance was considerably smaller than the one used for lactic acid formation, and could therefore be neglected in the model. Though strictly speaking this is not true, this assumption is often made in lactic acid fermentations and complex bioprocesses [36,45,54]. A new model can then be obtained to describe the consumption of substrate as a function of lactic acid production. Excluding the growth and maintenance terms from equation 17 and integrating with initial conditions S_0 and P_0 gives

$$S_0 - S = \frac{1}{Y_{p/s}}\left(P - P_0\right) \tag{18}$$

In order to estimate $Y_{p/s}$ (g of lactic acid produced per g of glucose consumed) the glucose and lactic acid concentrations were determined at regular time intervals for a number of batch cultures. The results are shown in Figure 5 and indicate that the glucose uptake and lactic acid formation are linearly correlated (r = 0.99, *F*-value = 794.2). The estimated $Y_{p/s}$ value was 0.51±0.02 g g^{-1}, which is considerably lower than other reported values for LAB at optimum conditions with *pH* control and nutrition rich media [43,45,55]. Our fermentation media contain high amounts of sugars, which mean that the low $Y_{p/s}$ obtained is probably due to the lack of *pH* control. Low $Y_{p/s}$ values in environments without pH control have been previously reported [42].

Introducing the model for lactic acid production (11) into equation 18 a full model for sugar consumption can be obtained

$$S = S_0 - \frac{1}{Y_{p/s}}\left\{\frac{\alpha_p A}{1+e^{\left[2+\frac{4\mu_m}{A}(\lambda-t)\right]}} + \frac{\beta_p A^2}{4\mu_m}\cdot\ln\left[\frac{e^{\left[2+\frac{4\mu_m}{A}(\lambda-t)\right]}+e^{\left(\frac{4\mu_m t}{A}\right)}}{1+e^{\left[2+\frac{4\mu_m}{A}(\lambda-t)\right]}}\right]\right\} \quad (19)$$

Simulations of cereal fermentations

In order to evaluate the ability of the model to describe the biochemical changes taking place during fermentation of *L. plantarum*, 6 batch cultures in different cereal extracts were monitored. The buffering capacity and the reducing sugar content of the media were initially measured. Values of relative cell population, sugar concentration, lactic acid concentration and pH were used to calculate the primary and secondary parameters at a given time. The experimental data and the values predicted by the models are depicted in Figure 6. The continuous lines represent the predicted values and the points the experimental results. It is interesting to note that the buffering capacity of a particular cereal medium increases with the percentage of cereal extract, which is probably associated with an increase in its protein and ash content [56].

In wheat media (Figure 6A) both the buffering capacity and the initial sugar content were low (S = 4.05 g l^{-1}, B = 0.23 mmoles HCl pH^{-1} l^{-1}), and the predicted cell populations were 15% lower than the experimental ones at the end of fermentation. This is probably due to variables not taken into account in the models, *e.g.* specific nutrient requirements for *L. plantarum* like peptides or aminoacids. The rest of the variables were appropriately described by the models.

In the medium with 5% malt (Figure 6B) the initial sugar concentration and buffering capacity were 4.92 g l^{-1} and 0.23 mmoles HCl pH^{-1} l^{-1}, respectively. In this case all variables are properly modelled with the exception of lactic acid for which the predicted values are underestimated. This could be due to the presence of various fermentable sugars at high levels.

In the fermentations using 30% barley extract (Figure 6C; S = 5.15 g l^{-1}, B = 0.48 mmoles HCl pH^{-1} l^{-1}), 40% barley (Figure 6D; S = 6.30 g l^{-1}, B = 0.55 mmoles HCl pH^{-1} l^{-1}) and 20% malt extract (Figure 6E; S = 18.85 g l^{-1}, B = 0.56 mmoles HCl pH^{-1} l^{-1}) the predicted relative cell populations, lactic acid, sugar concentrations and *pH* are in accordance with the experimental data. The model slightly underestimated the lactic acid production in the 20% malt medium (Figure 6E).

When citric acid was added (Figure 6F; 30% barley with 2.2 g l^{-1} of citric acid) *L. plantarum* growth increased as well as the lactic acid production (when compared to Figure 6C, 30% barley only). This could be attributed to the increased buffering capacity of the medium (0.48 to 0.97 mmoles HCl pH^{-1} l^{-1}), which was also the reason for the higher final *pH* value (approximately 1 unit higher than in Figure 6C). In this case sugar concentration and *pH* are well described by the models but relative cell populations and lactic acid are slightly underestimated.

Conclusions

In this study a model was developed to predict the kinetics of cell growth, lactic acid production, *pH* drop and sugar consumption in non *pH*-controlled cultures of *L. plantarum* as a function of the initial sugar concentration and the buffering capacity. The model included four differential

equations with parameters that were estimated using non-linear regression analysis. Secondary modelling of the derived parameters showed that the maximum specific growth rate (μ_m) and the relative maximum cell concentration (A) depends on buffering capacity and sugar concentration. The effect of the sugar could be described assuming Monod kinetics, while for the effect of the buffering capacity a linear relationship was used. The lag phase (λ) was modelled as the inverse of the maximum specific growth rate. The growth associated lactic acid production parameter α_p was constant, while the non-growth associated production parameter β_p was a linear function of buffering capacity. Parameter k, expressing the constant of lactic acid dissociation to hydrogen ions, depends on the buffering capacity while the minimum pH parameter ($pH_{\min}$) was constant. The yield of lactic acid on sugar ($Y_{p/s}$) did not depend on sugar or buffering capacity.

The results from the validation studies indicate that the model could adequately describe the biochemical changes during *L. plantarum* growth in these cereal media with a relatively high buffering capacity, though in some cases the lactic acid and the relative cell populations were slightly underestimated. From an industrial point of view, this study is a first step for the production of new cereal-based functional foods using media with high concentrations of malt, barley and wheat extracts. Our work established a set of equations useful in the optimization of the cultures defined with the purpose of a large-scale production of potentially probiotic beverages. However, more studies are needed in order to determine if the proposed fermentation media exerts some protective effects in the human gut.

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FIGURE CAPTIONS

Figure 1: Maximum specific growth rate (μ_m) as a function of the initial glucose concentration of the medium (up) and as a function of the buffering capacity (down). The data points for μ_m are obtained from Table 3.

Figure 2: Maximum relative cell concentration (*A*) as a function of the initial glucose concentration of the medium (up) and as a function of the buffering capacity (down). The data points for μ_m are obtained from Table 3.

Figure 3: $\mu_m\lambda$ as a function of μ_m. The data points are obtained from Table 3.

Figure 4: Parameter *k* as a function of buffering capacity.

Figure 5: S_0-S as a function of P-P_0. The data points are obtained from 5 batch cultures.

Figure 6: Experimental data (symbols) and model predictions (lines) for *L. plantarum* in: (A) 20% wheat extract; (B) 5% malt extract; (C) 30% barley extract; (D) 40% barley extract; (E) 20% malt extract; and (F) 30% barley extract with 2.2 g l^{-1} citric acid. *X* (●), relative cell concentration; *L* (○), lactic acid concentration; *pH* (□) and *S* (◆), sugar concentration.

TABLE CAPTIONS

Table 1: Composition of the synthetic media used for the development of the model.

Table 2: Notation used with units.

Table 3: Optimum parameter values for the logistic model 3 describing *L. plantarum* growth in synthetic media of different buffering capacity and glucose concentration. The values are the means with the corresponding confidence intervals ($\alpha = 0.05$). *F*-values are the results of the *F*-Fisher test ($\alpha = 0.05$) for 3 model degrees freedom and 21-30 error degrees freedom. r = correlation coefficient between observed and predicted data.

Table 4: Parameters for the secondary models expressing μ_m and *A* as a function of sugar concentration and buffering capacity. CI = confidence intervals ($\alpha = 0.05$). *F*-values are the results of the *F*-Fisher test ($\alpha = 0.05$) for 2 model degrees freedom and 6-8 error degrees freedom. r = correlation coefficient between observed and predicted data.

Table 5: Optimum parameters for equation 14 describing lactic acid production during *L. plantarum* growth in synthetic media of different buffering capacity and sugar concentration. The values are the means with the corresponding confidence intervals ($\alpha = 0.05$). *F*-values are the results of the *F*-Fisher test ($\alpha = 0.05$) for 2 model degrees freedom and 6-12 error degrees freedom. r = correlation coefficient between observed and predicted data. NS = not significant.

FIGURES

Figure 1

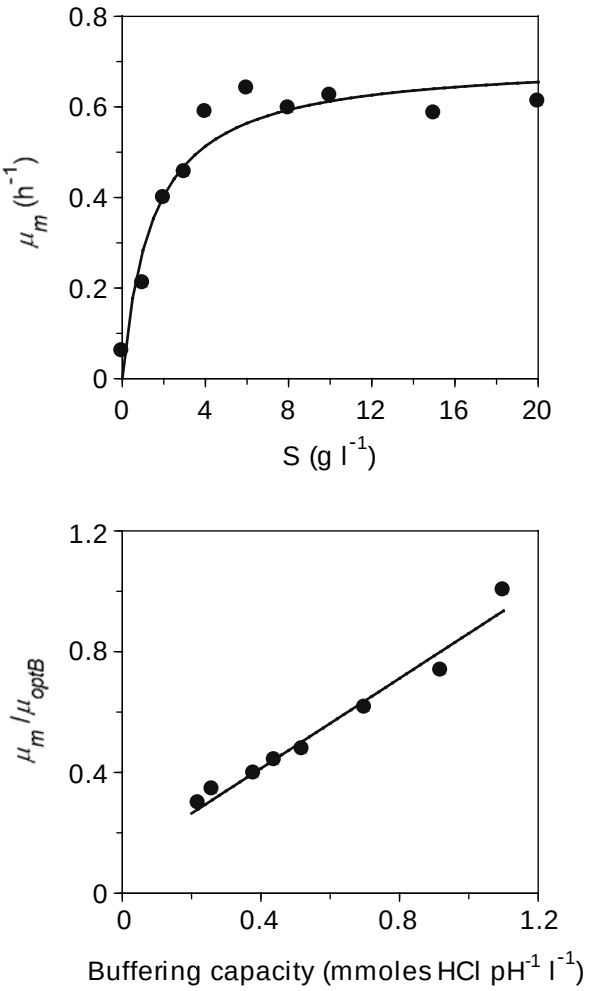


Figure 2

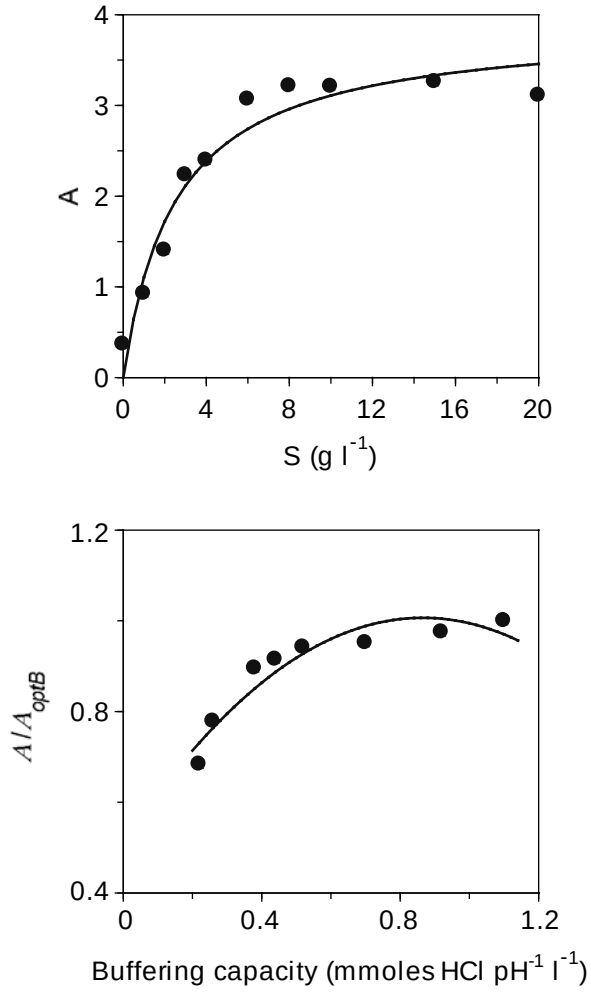


Figure 3

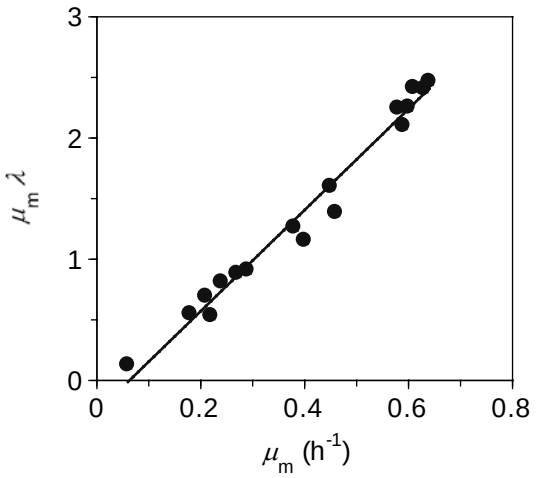


Figure 4

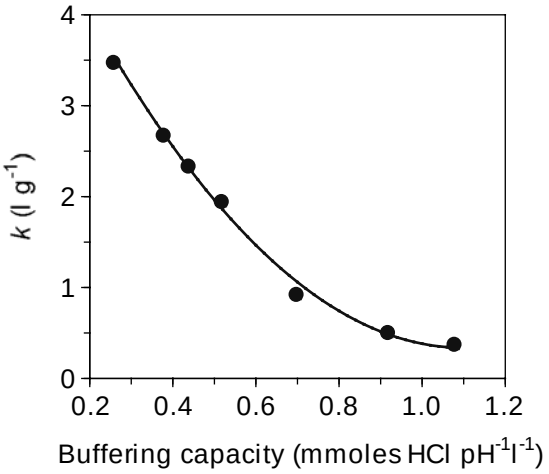

4
3
2
1
0
k (l g-1)
0.2
0.4
0.6
0.8
1.0
1.2
Buffering capacity (mmoles HCl pH-1l-1)

Figure 5

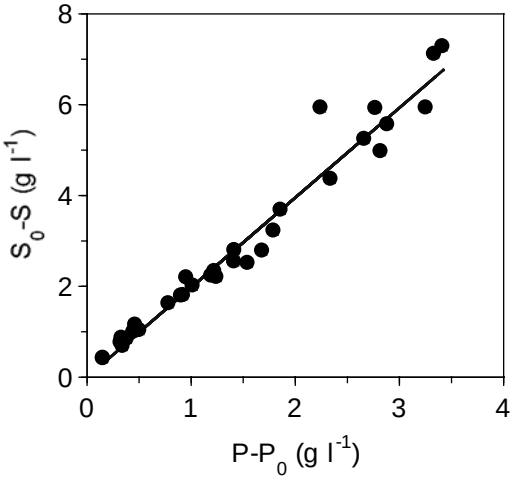
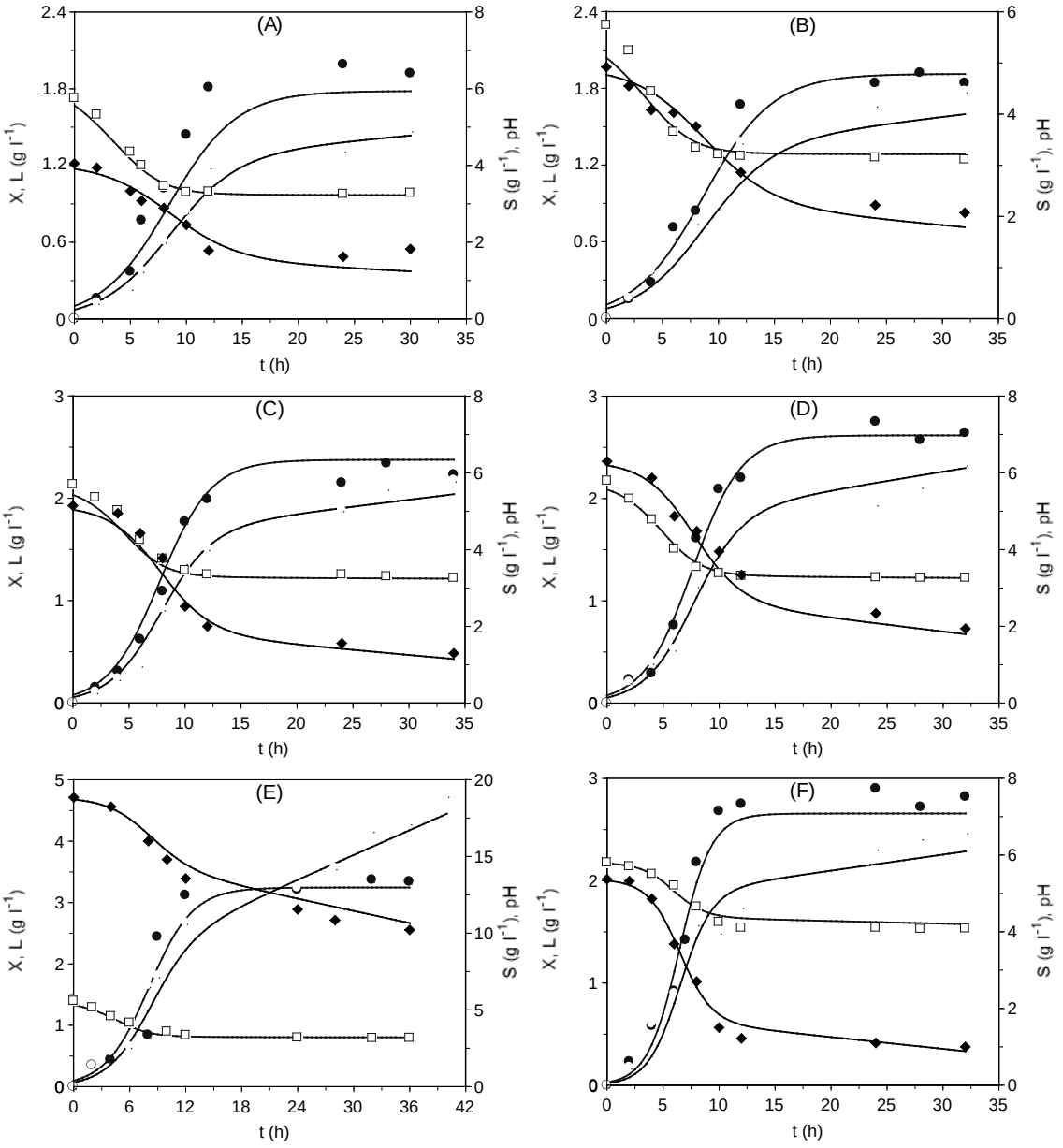


Figure 6



TABLES

Table 1

Controlling factor	Nutrients concentrations [a]
Buffering capacity (mmoles HCl pH^{-1} l^{-1}): 0.22, 0.26, 0.39, 0.44, 0.53, 0.70, 0.92, 1.09	FAN: 150 mg l-1 Glucose: 20 g l-1
Glucose concentrations (g l^{-1}): 0, 1.2, 3, 4, 6, 8, 10, 15, 20	FAN: 150 mg l-1 Buffering capacity: 1.09 mmoles HCl pH^{-1} l^{-1}

[a] All the media contained 5 g l^{-1} of yeast extract, 2 g l^{-1} Lab Lemco, 0.03 g l^{-1} $MnSO_4.4H_2O$, 0.1 g l^{-1} $MgSO_4.7H_2O$ and 1 ml l^{-1} Tween 80.

Table 2

X :	Biomass as relative cell population, dimensionless ($\log_{10}(N/N_0)$)
t :	Time, h
N :	Cell concentration, cfu ml^{-1}
N_0 :	Initial cell concentration, cfu ml^{-1}
μ_m :	Maximum specific growth rate, h^{-1}
λ :	Growth lag phase, h
A :	Maximum relative cell population, $\log_{10}(N/N_0)_{max}$
μ_{optS} :	Maximum specific growth rate at the optimum sugar concentration, h^{-1}
K_S :	Half-saturation constant, g l^{-1}
S :	Sugar concentration, g l^{-1}
S_0 :	Initial sugar concentration, g l^{-1}
B :	Buffer capacity, mmoles HCl pH^{-1} l^{-1}
α_1 :	Regresion parameter, dimensionless
β_1 :	Regresion parameter, pH l $mmoles^{-1}$ HCl
μ_{opt} :	Maximum specific growth rate at the conditions where S and B are at their optimum, h^{-1}
A_{optS} :	Maximum A value at the optimal glucose concentration, dimensionless
K'_S :	Half-saturation constant, g l^{-1}
A_{optB} :	Maximum A value at the optimal buffer capacity, dimensionless
α_2 :	Regresion parameter, dimensionless
β_2 :	Regresion parameter, pH l $mmoles^{-1}$ HCl
c_2 :	Regresion parameter, pH^2 l^2 $mmoles^{-2}$ HCl
A_{opt} :	Maximum A value at the conditions where S and B are at their optimum, dimensionless
α_3 :	Regresion parameter, dimensionless
β_3 :	Regresion parameter, h
P :	Lactic acid concentration, g l^{-1}
P_0 :	Initial lactic acid concentration, g l^{-1}
α_p :	Growth associated constant, g lactic acid $(\log_{10}(N/N_0))^{-1}$ l^{-1}
β_3 :	Non-growth associated constant, g lactic acid $(\log_{10}(N/N_0))^{-1}$ l^{-1} h^{-1}
k :	Constant, g l^{-1}
$Y_{p/s}$:	Yield of lactic acid production on substrate, g (lactic acid) g^{-1} (sugar)
$Y_{x/s}$:	Relative cell population yield on substrate, $\log_{10}(N/N_0)$ g^{-1} (sugar) l
m_s :	Relative cell maintenance coefficient, g (sugar) l^{-1} $[\log_{10}(N/N_0)]^{-1}$ h^{-1}

Table 3

Buffering capacity	μ_m	*A*	λ	Data points	*F*-value	r
0.22	0.182±0.026	2.125±0.102	3.008±0.840	33	1101.2	0.988
0.26	0.210±0.035	2.420±0.122	3.293±0.955	24	955.4	0.991
0.39	0.242±0.052	2.784±0.181	3.346±1.231	24	570.3	0.984
0.44	0.269±0.038	2.845±0.129	3.274±0.805	33	1155.6	0.989
0.53	0.291±0.044	2.928±0.144	3.121±0.837	33	1025.1	0.987
0.70	0.375±0.067	2.958±0.164	3.367±0.830	33	829.2	0.983
0.92	0.450±0.044	3.031±0.083	3.550±0.382	33	3664.9	0.996
1.09	0.612±0.087	3.109±0.084	3.943±0.418	33	2921.0	0.995
Sugar	μ_m	*A*	λ	Data points	*F*-value	r
0	0.061±0.023	0.367±0.031	2.063±1.258	10	392.6	0.991
1	0.211±0.077	0.925±0.051	2.520±0.895	30	615.2	0.970
2	0.399±0.088	1.403±0.045	2.891±0.394	25	1766.9	0.992
3	0.456±0.062	2.232±0.049	3.033±0.363	33	3915.9	0.995
4	0.589±0.141	2.394±0.088	3.564±0.504	29	1395.2	0.990
6	0.641±0.047	3.067±0.044	3.845±0.193	29	10040.5	0.999
8	0.597±0.063	3.214±0.074	3.772±0.331	29	3995.3	0.997
10	0.625±0.043	3.208±0.046	3.845±0.203	27	9785.6	0.999
15	0.586±0.054	3.259±0.067	3.829±0.309	42	4908.3	0.996
20	0.612±0.087	3.109±0.084	3.943±0.418	33	2921.0	0.995

Table 4

Controlling factor	Equation	Parameter	Value ± CI	*F*-value	r
Glucose	4	μ_{optS} (h^{-1})	0.703±0.099	405.0	0.965
		K_s (g l^{-1})	1.482±0.963		
Buffering capacity	5	μ_{optB} (h^{-1}), from table 2	0.612±0.087	623.04	0.983
		α_1	0.540±0.041		
		β_1 (pH l mmoles^{-1} HCl)	0.745±0.138		
Glucose	Similar to 4	A_{optS}	3.893±0.632	429.9	0.973
		K'_s (g l^{-1})	2.519±1.420		
Buffering capacity	8	A_{optB} , from table 2	3.109±0.084	1378.2	0.951
		α_2	0.949±0.056		
		β_2 (pH l mmoles^{-1} HCl)	0.391±0.147		
		c_2 (pH^2 l^2 mmoles^{-2} HCl)	-0.661±0.508		

Table 5

Buffering capacity	α_p	β_p	Data points	*F*-value	r
0.22	0.781±0.081	0.022±0.005	14	1623.7	0.994
0.26	0.691±0.062	0.021±0.004	14	2757.3	0.997
0.39	0.747±0.086	0.014±0.005	12	1257.0	0.996
0.44	0.643±0.074	0.023±0.005	14	2220.4	0.997
0.53	0.841±0.078	0.006±0.005	13	1340.6	0.994
0.70	0.722±0.075	0.016±0.005	13	1638.0	0.997
0.92	0.713±0.074	0.023±0.005	13	1736.4	0.996
1.09	0.752±0.032	0.021±0.002	13	8316.2	0.999
Sugar	α_p	β_p	Data points	*F*-value	r
1	0.420±0.092	0.000 (NS)	8	119.6	0.963
2	0.702±0.060	0.000 (NS)	10	1049.4	0.991
3	0.707±0.019	0.003±0.001	10	9817.6	0.999
4	0.667±0.170	-0.001 (NS)	10	114.0	0.981
6	0.720±0.049	0.007±0.003	11	2236.2	0.997
8	0.745±0.145	0.013±0.008	11	341.5	0.985
10	0.822±0.103	0.012±0.006	11	705.1	0.993
15	0.715±0.123	0.015±0.007	10	489.7	0.990
20	0.752±0.032	0.021±0.002	13	8316.2	0.999