Modelling the Combined Effect of pH and Acetic Acid on the Growth Rate of Escherichia coli

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Abstract

In recent years, the food industry has aimed to achieve food safety through milder combinations of multiple treatments/storage conditions. In this way, mathematical models that accurately describe the combined effect of environmental conditions on microbial growth dynamics are of great interest. Independent factors can be related in a multiplicative way to describe the combined effect (gamma hypothesis). However, at more stressful conditions, these factors might interact. In this research, the influence of pH and acetic acid on the growth rate of Escherichia coli K12 is modelled. To this end, a set of computer controlled bioreactor experiments was performed under static environmental conditions. Parameter estimations were performed directly on the cell density measurements using pH and undissociated acid models recovered from literature. The best-fitting structures were combined in a gamma model and its validity was assessed by comparison with the synergistic models of Augustin and Carlier [1], Le Marc et al. [2] and the gamma-interaction model of Akkermans et al. [3].

The presence of high amounts of acetic acid in the broth led to lower growth rates in the suboptimal pH range. Consequently, the individual effect of pH could not be determined. Given the current dataset, the occurrence of interactions is highly questionable. The small improvement in the quality-of-fit obtained with the gamma-interaction model is not sufficient to refute the gamma hypothesis. More pronounced interactions are expected at lower pH values and higher undissociated acid concentrations, where no data was available. Therefore, future research focused on these conditions is required.

Keywords: Microbial growth rate; organic acids; predictive microbiology; bioreactor experiments; Escherichia coli

1. Introduction

Quality and shelf life of food products are often compromised during their life cycle by the presence and growth of bacteria. A large variety of foods (e.g., undercooked meat, potatoes, acidic foods, raw milk, yogurt and fermented sausages) has been associated to foodborne illnesses and human infections [4].

Outbreaks of foodborne pathogens can have huge human and economic impacts. The Centers for Disease Control and prevention (CDC) reported that 5-10% of the diagnosed Shiga toxin-producing Escherichia coli (STEC) infections evolve to a fatal hemolytic-uremic syndrome (HUS), which can cause a fatal renal failure [5]. Most of the these cases have been caused by the serotype O157:H7 [6]. In 2011, an infection caused by STEC O104:H4 affected 3.950 people in Europe, causing death to 53 HUS patients and losses of around 200 million euros a week for the Spanish economy [7].

It is, therefore, of increased interest to quantify the effects of processing, storage and distribution on the growth of spoilage microorganisms and food pathogens. In predictive microbiology, microbial knowledge is combined with mathematical equations to build predictive models for both the evolution of microorganisms (primary models) and the influence of the environmental conditions on microbial growth dynamics (secondary models).

Organic acids have recognized antimicrobial properties for a wide spectrum of spoilage bacteria and food pathogens for being effective even at low concentrations and by contributing to decrease the extracellular pH to growth-preventing values. Acetic acid is a popular food preservative used in commercial mayonnaise, vinegar, salad dressings and sauces. The inhibitory effect of organic acids results mainly from their
undissociated form, which can enter the cytoplasm of microbial cells and reduce the intracellular pH [8]. Previous studies on modelling the combined effect of pH and organic acids assume that these are independent and can, therefore, be combined in a multiplicative way to describe the overall effect on the microbial growth rate [8-10]. At more stressful conditions, however, it is possible that synergistic interactions between factors produce a combined effect which is greater than expected [1-3].

The present case study aims to: (i) demonstrate the need for multiplicative models that describe the maximum specific growth rate as a function of pH and acetic acid concentration; (ii) compare the multiplicative combined effect model with three synergistic models. To this end, a set of 26 bioreactor experiments with E. coli K12 was performed under static conditions. The multiplicative combination of pH and acetic acid was modelled according to the gamma hypothesis and compared to the interaction models of Augustin and Carlier [1], Le Marc et al. [2] and the very recently proposed gamma-interaction model of Akkermans et al. [3], applied in this research, for the first time, to the effects of pH and organic acid concentration.

2. Materials and methods

2.1. Bacterial strain and media used

E. coli K12 MG1655 (CGSC#6300) was obtained from the Coli Genetic Stock Center at Yale University. A stock culture in Brain Heart Infusion broth (BHI, Oxoid/VWR) was supplemented with 20% (w/v) glycerol (Acros Organics) and stored at -80°C before the experiments and -20°C during the experiments. In all cases, E. coli K12 MG1655 was grown in BHI broth (37 g/L) and the plates for cell counting were prepared with BHI agar (BHIA, BHI supplemented with 14 g/L technical agar nr. 3, Oxoid).

2.2. Bacterial strain and media used

The inoculum was prepared from a single colony grown on a BHI agar plate. At first, a loopful of stock culture was spread onto a BHI plate and stored in a controlled incubator (model KBP6151, Termaks) at 37°C overnight. Then, a single colony was transferred from this plate into a first 50 mL Erlenmeyer containing 20 mL of fresh BHI and incubated at 37°C for 9 h. Finally, 20 µL of the stationary phase culture was transferred to a second 50 mL Erlenmeyer also containing 20 mL fresh BHI. At last, the second Erlenmeyer was incubated at 37°C for 15 h previously to inoculation.

2.3. Bioreactor experiments

Experiments were performed in a set of computer controlled bioreactors (BioStat B, Sartorius Stedim 336 GmbH). The reactor vessels had a total volume of 5 L and were, in all cases, filled with 3 L of BHI broth. Acetic acid (Acros Organics, New jersey, pKa 4.75) was added to the BHI broth in different amounts (see section 3.5) and autoclaved following the manufacturer’s instructions.

Temperature was measured with a PT100 resistance temperature sensor and controlled to 37°C by a proportional-integral-derivative (PID) controller and a circulation chiller. pH measurements were performed using a gel-filled pH electrode (Hamilton Company), and were corrected for temperature. pH was kept constant for each experiment by adding acid (0.5 M H₂SO₄, Sigma-Aldrich) or base (1 M KOH, Thermo Fisher Scientific) solutions. Aeration was set to 0.2 L/min after autoclaving. Dissolved oxygen concentration was controlled at the stabilized level during the experiment. The vessel content was stirred with Rushton impellers at a speed of 75 rpm. The total amount of acetic acid in the growth medium was determined by performing High-Performance Liquid Chromatography (HPLC) measurements on samples taken from the bioreactor after setting the controls (performed by other personnel from the laboratory). The Henderson-Hasselbalch equation (equation 1) was used to convert the total concentration of acetic acid, [Ac] measured [ppm], to the concentration of the undissociated form [HAc].

\[
[HAc] = \frac{[Ac]_{\text{measured}}}{1 + 10^{pH-(pK_a)}}
\] (1)

1 mL of antifoaming agent (Y-30 emulsion, Sigma-Aldrich) was added prior to every experiment to prevent foaming.

2.4. Sampling and microbiological analysis

A sample was aseptically taken from the bioreactor at regular time intervals (every one or two hours, depending on the specific experimental conditions) to determine the cell density via plate counting. All samples were taken during daytime. To obtain countable plates, the appropriate dilutions were made using 900 µL of stock NaCl solutions (0.8% (w/v), Sigma-Aldrich). 20 µL of each dilution was then plated onto BHIA plates, in six replicates. These plates were incubated at 37°C for approximately 15 h. Then, viable cell numbers (CFU/mL) were determined by plate counting and the average value over the six replicates was used as the cell density measurement for the considered sample. Depending on the experimental conditions applied to the bioreactor, experiments lasted between 15 and 35 h.

2.5. Experimental design

An overview of the experimental design is represented in Figure 1. Dataset 1 is the set of 11 experiments used to model the separate effect of pH by not adding acetic acid to the medium. Dataset 2 is the set of 5 experiments performed at pH 6 used to assess the effect of acetic acid concentration on the
maximum specific growth rate of _E. coli_ K12. Dataset 3 includes the experiments from dataset 1, dataset 2 and a set of experiments performed at suboptimal pH conditions and different acetic acid concentrations. This dataset of 26 experiments was used to model the combined effects of pH and acetic acid concentration and focuses on suboptimal conditions, as these are more relevant to the food industry than superoptimal conditions.

![Figure 1: pH [\(\cdot\)] and total acetic acid concentration, \([Ac] \text{ [ppm]}\), for the 26 bioreactor experiments. Dataset 1 (11 experiments): pH effect (O). Dataset 2 (4 experiments): undissociated acid effect (+). Dataset 3 (26 experiments): interactions (\(x\)).](image)

### 2.6. Mathematical Modelling


The extensively used primary model of Baranyi and Roberts [11] was used to describe the microbial population \([\text{ln(CFU/mL)}]\) as a function of time \([\text{h}]\).

\[
\frac{dn(t)}{dt} = \frac{1}{1+\exp(-q(t))} \mu_{\text{max}} \cdot (1 - \exp(n(t) - n_{\text{max}}))
\]

\[
\frac{dq(t)}{dt} = \mu_{\text{max}}
\]

\(n(t) \text{ [ln(CFU/mL)]}\) is the natural logarithm of cell density and \(q(t) \text{ [\(\cdot\)]}\) is a measure of the physiological state of cells, at time \(t\). \(n_0\) and \(n_{\text{max}}\) are the normal logarithms of the initial cell concentration and of the maximum cell density, respectively. \(\mu_{\text{max}}\) is the maximum specific growth rate \([1/\text{h}]\). The effect of pH and acetic acid on \(\mu_{\text{max}}\) is obtained by combining the primary model of Baranyi and Roberts [11] with secondary models recovered from literature.

#### 2.6.2. Secondary modelling: pH effect

The model structures used to model the effect of pH on the maximum specific growth rate, \(\mu_{\text{max}}\), were the Cardinal pH Model (CPM) proposed by Rosso et al. [12] and its adaptations: srCPM and aCPM [3] (see Table 1). All these models have four parameters which are biologically interpretable. These are the theoretical minimum, optimum and maximum pH for growth \((pH_{\text{min}} \cdot pH_{\text{opt}} \cdot pH_{\text{max}}\) \(\cdot \mu_{\text{opt}} \text{ [h]}\), the maximum specific growth rate at optimum pH conditions. The srCPM of Akkermans et al. [3] includes an additional parameter, \(k\). Although this parameter has an exclusively structural correlation, it is associated with ability of _E. coli_ cells to maintain their cytoplasmic pH (pHi) approximately constant, despite changes in the environmental pH [13]. The results obtained by Baka et al. [14] suggest that this ability is particularly efficient in the external pH range 6.5-8.5 for _E. coli_ K12. In the aCPM [3], the suboptimal pH factors are raised to the power \(\eta\), to account for the different responses in the suboptimal and superoptimal pH regions. In all three cardinal pH models, it is assumed that the growth rate is equal to zero for pH values lower than \(pH_{\text{min}}\) and higher than \(pH_{\text{max}}\). To facilitate computations, \(k\) and \(\eta\) were fixed to different values, instead of being estimated along with the other model parameters.

<table>
<thead>
<tr>
<th>Model</th>
<th>Formula</th>
<th>Nr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPM</td>
<td>[\mu_{\text{max}}(pH) = \mu_{\text{opt}} \cdot \left(\frac{(pH - pH_{\text{min}}) \cdot (pH - pH_{\text{max}})}{(pH - pH_{\text{min}}) \cdot (pH - pH_{\text{max}}) - (pH - pH_{\text{opt}})^2}\right)]</td>
<td>(4)</td>
</tr>
<tr>
<td>srCPM</td>
<td>[\mu_{\text{max}}(pH) = \mu_{\text{opt}} \cdot \left(\frac{(pH - pH_{\text{min}}) \cdot (pH - pH_{\text{max}})}{(pH - pH_{\text{min}}) \cdot (pH - pH_{\text{max}}) - (pH - pH_{\text{opt}})^2}\right)^{1/k}]</td>
<td>(5)</td>
</tr>
<tr>
<td>aCPM</td>
<td>[\mu_{\text{max}}(pH) = \mu_{\text{opt}} \cdot \left(\frac{(pH - pH_{\text{min}}) \cdot (pH - pH_{\text{max}})}{(pH - pH_{\text{min}}) \cdot (pH - pH_{\text{max}}) - (pH - pH_{\text{opt}})^2}\right)^{1/k}]</td>
<td>(6)</td>
</tr>
</tbody>
</table>

#### 2.6.3. Secondary modelling: Undissociated acetic acid effect

Presser et al. [8] has modelled the effect of lactic acid concentration, \([HAc] \text{ [ppm]}\), on the growth rate of _E. coli_ M23 assuming that the und dissociated form of the acid presents a higher inhibitory potential than the dissociated form under low pH conditions and that \(\mu_{\text{max}} \text{ [h]}\) decreases linearly with the increase in the concentration of the und dissociated form of organic acids. Le Marc et al. [2] proposed a model structure where the linear relationship of the growth rate was with the square root of the concentration of und dissociated acetic acid instead. In this research, no previous \(\mu_{\text{max}} - [HAc]\) relationship was initially assumed. Instead, a shape parameter \(\alpha \text{ [\(\cdot\)]}\) was introduced and estimated along with the other model parameters to provide a better fit to the data. To study the influence of this parameter on the model output, the two following structures were proposed:
\[
\mu_{\text{opt}} = (1 - \frac{\mu_{\text{opt}}}{\mu_{\text{opt}}})^\beta
\]

\[
\mu_{\text{max}}(\text{[HAc]}) = \mu_{\text{opt}} (1 - \frac{\text{[HAc]}}{\text{MIC}})^\beta
\]

\[
\mu_{\text{max}}(\text{[HAc]}) = \mu_{\text{opt}} (1 - \frac{\text{[HAc]}}{\text{MIC}})^\beta
\]

\[
\mu_{\text{opt}} \text{ [h}^{-1}\text{]} \text{ is the maximum specific growth rate under optimum conditions, which corresponds to the absence of acetic acid from the medium at the set pH value, in this case. The MIC is the smallest concentration of an acid capable of inhibiting growth. Therefore, the growth rate is assumed to be equal to zero for undissociated acetic acid concentrations higher than the MIC.}

2.6.4. Gamma model

The combined effects of pH and undissociated acetic acid concentration were described by a gamma model. This hypothesis states that environmental factors act independently on microbial growth, so that the overall model structure results from the simple multiplication of these factors \([9, 15, 16]\). Equation 9 represents the gamma hypothesis for the environmental conditions considered in this case study.

\[
\mu_{\text{max}}(\text{pH,[HAc]}) = \mu_{\text{opt}} \cdot \gamma_{\text{pH}}(\text{pH}) \cdot \gamma_{\text{[HAc]})(\text{[HAc]})}
\]

The gamma factors \(\gamma_{\text{pH}}(\text{pH}) = \mu_{\text{max}}(\text{pH}) / \mu_{\text{opt}} \) and \(\gamma_{\text{[HAc]})(\text{[HAc]}) = \mu_{\text{max}}(\text{[HAc]}) / \mu_{\text{opt}}\) are given by the separate effect models from sections 2.6.2 and 2.6.3, respectively, and express the partial reduction of \(\mu_{\text{max}}\) caused by non-optimal values of pH or [HAc]. The validity of the gamma hypothesis for the current dataset was evaluated by comparison with three synergistic models: Augustin and Carlier \([1]\), Le Marc et al. \([2]\) and the gamma-interaction model of Akkermans et al. \([3]\).

2.6.5. Model of Augustin and Carlier

The model of Augustin and Carlier \([1]\) considers the existence of interactions between different environmental conditions by estimating new minimum cardinal parameters and including them in the multiplicative gamma model. The new suboptimal region parameters, \(p_{\text{H_{min,new}}}[\%]\) and \(MIC_{\text{new}} \text{ (ppm)}\), are determined by equations 10 and 11, respectively. \(\beta \text{ [\%]}\) is a shape parameter related to the extent of the interactions. Lower values of \(\beta\) result in smaller growth regions and, consequently, in more pronounced interactions \([3]\). Augustin and Carlier \([1]\) set \(\beta = 3 \text{ based on a set of published data. In this research, \(\beta\) was estimated to provide a better fit to data.}

\[
pH_{\text{min,new}}(\text{[HAc]}) = pH_{\text{opt}} - (\text{pH}_{\text{opt}} - pH_{\text{min}}) (1 - \frac{\text{[HAc]}}{\mu_{\text{MIC}}} )^\beta
\]

\[
MIC_{\text{new}}(\text{pH}) = MIC \cdot (1 - \frac{\text{pH}_{\text{opt}} - \text{pH}_{\text{min}}}{\text{pH}_{\text{opt}} - \text{pH}_{\text{min}}})^\beta
\]
are the interactions between the studied environmental conditions. In the absence of interactions, \( \beta \) is set to zero and the model equation is basically reduced to that of the gamma hypothesis. In this way, this model structure can also be applied over a sequential modelling approach, allowing the separate effects to be identified in the combined effect model [3]. In this research, \( \beta \) was estimated to provide a better fit to data.

2.6.8. Parameter estimation and confidence intervals

Parameters were estimated by the one-step method, i.e., directly on the cell density data by combining the primary model with the secondary models. The optimal combination of parameters was estimated from the set of experimental data via the minimization of the sum of squared errors (SSE). For that end, the lsqnonlin routine of the Optimization Toolbox of Matlab version 7.14 (The Mathworks Inc.) was used. All the ordinary differential equations were solved using the Matlab function ode45. The SSE for a single experiment with \( N \) measurements is calculated as follows:

\[
SSE = \sum_{i=1}^{N} (n_{m,i}(t_i) - n_{p,i}(t_i,p))^2
\]  

(20)

\( n_{m,i}(t_i) \) is the logarithm of the cell density measurement and \( n_{p,i}(t_i,p) \) the model prediction at time \( t_i \). \( p \) is a vector that contains the full set of parameters. The asymptotic (1-\( \alpha \))% confidence intervals for every estimated parameter \( p_i \) were determined from the Student’s t-distribution and the parameter variance \( s_{p_i}^2 \) (equation 21). \( s_{p_i}^2 \) was obtained from the main diagonal of the parameter covariance matrix \( V \) (equation 22).

\[
[ p_i \pm t_{(1-\alpha/N_p)} \sqrt{s_{p_i}^2} ]
\]  

(21)

\[
s_{p_i}^2 = V_{(i,i)}
\]  

(22)

The covariance matrix \( V \) can be approximated by the inverse of Fisher Information Matrix \( F \) [17]:

\[
V = F^{-1}
\]  

(23)

In turn, the Fisher Information matrix can be obtained from the mean sum of squared errors (MSE) and the Jacobian matrix \( J \), according to equation 24. The MSE (equation 25) is used in this research as a measure of the quality-of-fit to the experimental data. Lower values of the MSE are characteristic of a better quality-of-fit.

\[
F = \frac{1}{MSE} J^T J
\]  

(24)

\[
MSE = \frac{SSE}{N_e-N_p}
\]  

(25)

3. Results and discussion

3.1. Undissociated acetic acid in the growth medium

In Figure 2 is represented the set of pH-[HAc] conditions effectively applied to the bioreactor, as determined from the HPLC analysis and the Henderson-Hasselbalch equation on the total concentration of acetic acid. Considerably high amounts of the undissociated acid were shown to be present in experiments performed at low pH. This fact is more relevant for experiments where only the effect of pH should be present (dataset 1).

Figure 2: pH [-] and undissociated acetic acid concentration, [HAc] [ppm], for the 26 bioreactor experiments. Dataset 1 (11 experiments): pH effect (O). Dataset 2 (4 experiments): undissociated acid effect (+). Dataset 3 (26 experiments): interactions (X).

3.2. Modelling the effect of pH

To model the separate effect of pH on the maximum specific growth rate, the 11 experiments of dataset 1 (Figure 2) were considered. The model structures used to model this effect were the Cardinal pH Model (CPM) proposed by Rosso et al. [12] and its adaptations: srCPM and aCPM [3].

In the CPM [12], a small number of biologically interpretable parameters led to simple initial values estimations and to an immediate convergence using the lsqnonlin routine. However, with the srCPM [3], parameter estimation becomes more difficult due to the inclusion of a shape parameter \( k \). For this reason, instead of estimating \( k \) along with the other model parameters, this was given different values to determine the best fit on the E. coli growth data. Comparing the MSE values in Table 2, the quality-of-fit seems to have improved when \( k \) was raised from 2 to 3. Further increases, however, did not reveal to be useful in lowering the MSE value.
Table 2: Mean sum of squared errors for different $k$ values in the equation of the srCPM [3].

<table>
<thead>
<tr>
<th>$k$</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSE</td>
<td>0.183</td>
<td>0.092</td>
<td>0.131</td>
<td>0.199</td>
<td>0.286</td>
</tr>
</tbody>
</table>

The adaptation provided by the srCPM [3] accounts for the capacity *E. coli* cells to maintain pH homeostasis within a specific range, where $\mu_{\text{max}}$ remains practically constant despite changes in the environmental pH. For *E. coli* K12, the results obtained by Baka et al. [14] suggest that this plateau in a $\mu_{\text{max}}$ – pH plot should be observed in the pH range 6.50-8.50 [14]. Higher $k$ values result in a more extended plateau in the optimal pH region, that might not correspond to the limitations presented by the pH homeostasis mechanisms, explaining why the MSE decreases for $k$ values higher than 3. In the aCPM [3], the factors for the suboptimal pH conditions are raised to a shape parameter $\eta$ in both numerator and denominator. The $\eta$ that provides the best description of microbial growth was also found by equating it to different values and evaluating the mean sum of squared errors of the resultant aCPM [3]. The value of $k$ was kept at 3, as the aCPM [3] also includes the adaptation to the flat optimum provided by the model structure of the srCPM [3]. As it is shown in Table 3, The aCPM [3] structure that presented the best quality-of-fit (i.e., lowest MSE value) to the cell density data was obtained for $\eta$ equal to 2.

Table 3: Mean sum of squared errors for different $\eta$ values in the equation of the aCPM [3].

<table>
<thead>
<tr>
<th>$\eta$</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSE</td>
<td>0.064</td>
<td>0.098</td>
<td>0.161</td>
<td>0.341</td>
</tr>
</tbody>
</table>

The growth rates calculated using the CPM [12], the best-fitting srCPM [3] and the best-fitting aCPM [3] models are plotted against the “experimental” growth rates, estimated with the model of Baranyi and Roberts [11] in Figure 3. The cardinal parameter estimates with the correspondent 95% confidence intervals, mean sum of squared errors and accuracy and bias factors for the three pH models are shown in Table 4.

As expected, the model structure of the CPM [12] was revealed to be unable to describe the effect of pH on *E. coli* growth rate. The accuracy and bias factors for this model also suggest that it leads to the least accurate predictions of the three cardinal pH models and results in the largest underestimations of the growth rate. Adapting the model structure to that of the srCPM [3], which admits the existence of pH homeostasis mechanisms, led to an MSE value 87% lower than that of the CPM [12]. A further improvement of the MSE is achieved by considering the different response of *E. coli* cells in the suboptimal and superoptimal range. In fact, the presence of the parameter $\eta$ in the model structure of the aCPM [3] reduced the MSE value obtained with the srCPM [3] in 30%.

Figure 3: Comparison between different secondary models for the effect of pH [-] on the maximum specific growth rate [1/h]: CPM ( — — ), srCPM ($k=3$) (—), aCPM ($k=3; \eta=2$) (— —). Model predictions are plotted against the $\mu_{\text{max}}$ estimates of the Baranyi and Roberts model with the 95% confidence bounds (x).

Table 4: Secondary model parameter estimates, 95% confidence intervals and mean square errors of the CPM [12], srCPM [3] and aCPM [3].

<table>
<thead>
<tr>
<th>Model</th>
<th>CPM</th>
<th>srCPM ($k=3$)</th>
<th>aCPM ($k=3; \eta=2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$pH_{\text{min}}$ [-]</td>
<td>4.29 ($\pm$0.12)</td>
<td>4.77 ($\pm$0.01)</td>
<td>4.62 ($\pm$0.02)</td>
</tr>
<tr>
<td>$pH_{\text{opt}}$ [-]</td>
<td>7.23 ($\pm$0.12)</td>
<td>7.47 ($\pm$0.06)</td>
<td>7.10 ($\pm$0.08)</td>
</tr>
<tr>
<td>$pH_{\text{max}}$ [-]</td>
<td>9.43 ($\pm$0.16)</td>
<td>9.11 ($\pm$0.03)</td>
<td>9.03 ($\pm$0.01)</td>
</tr>
<tr>
<td>$\mu_{\text{opt}}$ [h$^{-1}$]</td>
<td>2.35 ($\pm$0.08)</td>
<td>2.21 ($\pm$0.02)</td>
<td>2.13 ($\pm$0.02)</td>
</tr>
<tr>
<td>MSE</td>
<td>0.681</td>
<td>0.092</td>
<td>0.064</td>
</tr>
</tbody>
</table>

Evaluating the cardinal pH values is more difficult, as the amount of research on the growth boundaries for the specific *E. coli* strain K12 is very limited and these are also dependent on the type of acids present in the medium and in their respective concentration. In spite of this fact, the most accurate $pH_{\text{min}}$ estimation corresponds to a value of 4.77 and was obtained with the srCPM [3]. However, this value is unlikely to be realistic, as growth has been observed in an additional experiment performed at a lower pH of 4.65 (data not shown). The lack of “no-growth” data for pH values below pH 4.65 makes the $pH_{\text{min}}$ predictions for the aCPM [3] and, more specifically, the CPM [12] model structures an extrapolation of the growth boundaries. CPM [12] predictions resulted in the largest growth region of the three pH models tested and admit the occurrence of growth for pH values up to 9.50. Nevertheless, there is no experimental evidence supporting this. In an experiment performed with *E. coli* K12 at pH 9.50 and 40°C, Baka et al. [14] reported the induction of cell inactivation after 5 hours. Therefore, it is possible that $pH_{\text{max}}$ values such as that estimated by the CPM [12] are correct. However, as it was previously mentioned, experiments in that pH range would have to be performed to validate its value. Even though the influence of undissociated acetic acid on these results has to be taken into account in further parameter estimations, the best approximation to the real microbial response was obtained with the aCPM [3].
3.3. Modelling the effect of undissociated acetic acid

To assess the effect of undissociated acetic acid concentration on the maximum specific growth rate of *E. coli*, four different sets of experiments from Figure 2 were initially considered: (i) 6 experiments at pH 5.00, (ii) 4 experiments at pH 5.50, (iii) 5 experiments at pH 6.00 and (iv) 4 experiments at pH 6.50. Equations 7 and 8 were used to describe $\mu_{\text{max}}$ as a function of $[\text{HAc}]$. $\alpha$ is a structural parameter, estimated to provide the best fit to data. Figure 4 compares the model predictions of equations 7 and 8 with the growth rates estimated by the model of Baranyi and Roberts [11] (equations 7 and 8), at different pH values. The value estimated for $\alpha$, the minimum inhibitory concentration of undissociated acetic acid ($\text{MIC}$), the optimum growth rate and the MSE value are represented in Table 5 and Table 6 for equations 7 and 8, respectively.

Table 5: Estimated $\text{MIC}$, $\alpha$ and $\mu_{\text{opt}}$, with $\mu_{\text{max}}(\text{HAc}) = \mu_{\text{opt}}(1 - \frac{[\text{HAc}]}{[\text{MIC}]})^\alpha$. Each parameter has a 95% confidence interval associated to. The MSE is also shown.

<table>
<thead>
<tr>
<th>pH</th>
<th>5.00</th>
<th>5.50</th>
<th>6.00</th>
<th>6.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC</td>
<td>113.55 (± 23.67)</td>
<td>141.22 (± 16.84)</td>
<td>146.71 (± 5.70)</td>
<td>142.98 (± 11.26)</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>1.00 (± 2.55)</td>
<td>0.94 (± 0.61)</td>
<td>0.91 (± 0.13)</td>
<td>0.95 (± 0.12)</td>
</tr>
<tr>
<td>$\mu_{\text{opt}}$</td>
<td>1.93 (± 2.98)</td>
<td>1.98 (± 0.57)</td>
<td>2.04 (± 0.10)</td>
<td>2.11 (± 0.06)</td>
</tr>
<tr>
<td>MSE</td>
<td>1.510</td>
<td>0.716</td>
<td>0.152</td>
<td>0.099</td>
</tr>
</tbody>
</table>

Table 6: Estimated $\text{MIC}$, $\alpha$ and $\mu_{\text{opt}}$, with $\mu_{\text{max}}(\text{HAc}) = \mu_{\text{opt}}(1 - \frac{[\text{HAc}]}{[\text{MIC}]})^\alpha$. Each parameter has a 95% confidence interval associated to. The MSE is also shown.

<table>
<thead>
<tr>
<th>pH</th>
<th>5.00</th>
<th>5.50</th>
<th>6.00</th>
<th>6.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC</td>
<td>120.36 (± 97.81)</td>
<td>148.21 (± 65.47)</td>
<td>147.04 (± 23.46)</td>
<td>143.35 (± 32.43)</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>1.08 (± 2.35)</td>
<td>1.05 (± 0.95)</td>
<td>1.05 (± 0.33)</td>
<td>1.08 (± 0.36)</td>
</tr>
<tr>
<td>$\mu_{\text{opt}}$</td>
<td>1.79 (± 1.22)</td>
<td>1.87 (± 0.25)</td>
<td>1.97 (± 0.08)</td>
<td>2.11 (± 0.05)</td>
</tr>
<tr>
<td>MSE</td>
<td>1.029</td>
<td>0.598</td>
<td>0.220</td>
<td>0.092</td>
</tr>
</tbody>
</table>

Both the computed mean sum of squared errors and the estimated parameters confirm that the position of the mathematical parameter $\alpha$ on the model structure does not have a significant influence on its output, as $\alpha$ was estimated to be very close to 1.00 for every pH value. In all the following parameter estimations, $\alpha$ was fixed to 1.00 for an easier parameter estimation and to obtain a simpler model structure where only biologically interpretable parameters are included. An $\alpha$ value of approximately 1.00 supports the linear relationship between the growth rate reduction and the concentration of the undissociated form of the organic acid stated by Presser et al. [8].

An observation common for the results presented in both Table 5 and Table 6 is that the MSE consistently increases with lower pH values. This shows that growth might be also a function of pH in the sense that, at more stressful pH conditions, the capacity of this separate effect model to accurately describe the microbial response to $[\text{HAc}]$ decreases. However, this increase in the error with a decreasing pH could be due to either (i) variability in the behavior of the population resultant from the stressful conditions or (ii) low accuracy of the acetic acid measurements at low pH values. The fact that the experiments were performed with high numbers of cells in the bioreactor, with the total initial number being around 3,000,000 cells, makes it difficult to motivate that the growth rate of the entire population could really be variable at the exact same conditions. Therefore, the second statement is more likely to be correct, meaning that there are small errors in the acetic acid concentration measurements and that the measured growth rates are, in fact, at different concentrations of undissociated acetic acid. In the absence of interactions, changing the level of stress of one factor should not affect the cells resistance to the other. This implies that the value of the secondary model parameters should not change, even at more stressful conditions of the other factor. Indeed, the $\text{MIC}$ was consistently estimated with a value around 145.00 ppm for all moderate and slightly acid pH conditions. However, at pH 5.00, where pH is in the proximity of the lower growth boundary, a significantly lower value was obtained for this parameter with both model equations. It should be noted, however, that the estimated $\text{MIC}$ values are affected of a high uncertainty, most likely resultant from the existence of small errors in the acetic acid measurements at low pH values and are, therefore, not very reliable. A trend could also be observed for $\mu_{\text{opt}}$, as it slightly decreased with lower pH values. This is, however, more straightforward, since this parameter is defined as the growth rate for 0.00 ppm of acetic acid and is also dependent on the environmental pH, as seen by the decrease in MSE values. Therefore, it was expectable that its value would be higher for near optimal pH conditions.

Everything considered, the best-fitting model for the separate effect of the undissociated acetic acid concentration on the growth rate of *E. coli* is the one that minimizes the MSE, i.e., that provides the best description of the microbial response to changes in $[\text{HAc}]$. Therefore, this corresponds to the parameter estimates at pH 6.50 of Table 6.
3.4. Modelling the combined effect of pH and undissociated acetic acid

It is known from the HPLC measurements that high amounts of undissociated acetic acid were present in the broth of experiments performed at low pH conditions. To estimate the pure pH effect: (i) \( \gamma([HAc]) \) was determined from the acetic acid model of equation 8, at pH 6.50, and calculated for the undissociated acetic acid concentration that was measured at each pH, in experiments where no acetic acid was added; (ii) at different pH values, the \( \mu_{\text{max}} \) that was calculated based on the parameter estimation with Baranyi and Roberts [11] was divided by the \( \gamma([HAc]) \) calculated from the acetic acid model; (iii) this yielded a set of data points where effect of acetic acid was basically removed, leaving just the pH effect. The results are shown in Figure 5 and demonstrate that there is a considerable reduction of the growth rate due to the presence of relatively large amounts of undissociated acetic acid at low pH values.

Since, in the end, the individual effect of pH was not correctly determined (because of the presence of acetic acid in the broth), it is not adequate to include the aCPM model [3] obtained with the parameters of Table 4 in a gamma model, as this assumes that (i) the individual effects are actually identified and (ii) the gamma concept holds for the combination of pH and acetic acid concentration. Therefore, the best way to test the model for the combined effect of pH and [HAc] seemed to be a global parameter estimation, i.e., to estimate all the secondary model parameters of the full gamma model on all the data.

As a benchmark, in the gamma model of equation 18, \( \gamma(pH) \) was initially described by the aCPM [3] and \( \gamma([HAc]) \) by the undissociated acetic model of Presser et al. [8] (equation 8 with \( \alpha \) equal to 1.00). Just as in section 3.2, \( k \) and \( \eta \) were given different fixed values to determine the best fit to the experimental data. Then, the resultant gamma model was compared to another one, where \( \gamma(pH) \) was described by the srCPM [3], to see if the description of the combined effect could be improved by taking a different gamma factor for the pH effect, particularly one that provides a more extended plateau in the suboptimal pH range. The results from Table 7 and Table 8 show that the gamma model for the combined effect is improved by using the
srCPM [3] as $\gamma(pH)$ and that the mean sum of squared errors is now minimized when $k$ is equalled to 4. This difference can be explained by the fact that in a combined effect model, it is no longer assumed that there is no acetic acid in experiments where it was not added to the broth, resulting in a different description of the effect of pH, such as when the effect of acetic acid was removed from experiments at different pH values.

Table 7: Comparison of the MSE value obtained with the non-synergistic gamma model, for a $\gamma(pH)$ given by the aCPM [3] or the srCPM [3] models obtained with different $k$ values. $\gamma$ was fixed to 2 in the model where the aCPM [3] is used to describe the pH effect.

<table>
<thead>
<tr>
<th>$k$</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSE_{aCPM}</td>
<td>0.466</td>
<td>0.428</td>
<td>0.323</td>
<td>0.356</td>
<td>0.365</td>
</tr>
<tr>
<td>MSE_{srCPM}</td>
<td>0.461</td>
<td>0.318</td>
<td>0.294</td>
<td>0.302</td>
<td>0.315</td>
</tr>
</tbody>
</table>

Table 8: Comparison of the MSE value obtained with the non-synergistic gamma model, for a $\gamma(pH)$ given by the aCPM [3] model obtained with different $\eta$ values. $\eta$ was fixed to 4.

<table>
<thead>
<tr>
<th>$\eta$</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSE_{aCPM}</td>
<td>0.323</td>
<td>0.504</td>
<td>0.522</td>
</tr>
</tbody>
</table>

To search for synergies between pH and $[HAc]$, the gamma hypothesis was compared to the models of Augustin and Carlier [1], Le Marc et al. [2] and the gamma-interaction model [3], based on dataset 3 of Figure 2. The estimated parameters and the resultant MSE values are displayed in Table 9.

Table 9: Parameter estimates, 95% confidence intervals and MSE values for the non-synergistic gamma model, the synergistic models of Augustin and Carlier [1], Le Marc et al. [2] and the gamma-interaction model of Akkermans et al. [3].

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$pH_{min}$ [\text{-}]</td>
<td>4.64 \ (\pm 0.05)</td>
<td>4.39 \ (\pm 0.14)</td>
<td>4.65 \ (\pm 0.05)</td>
<td>4.36 \ (\pm 0.37)</td>
</tr>
<tr>
<td>$pH_{opt}$ [\text{-}]</td>
<td>7.63 \ (\pm 0.13)</td>
<td>7.70 \ (\pm 0.13)</td>
<td>7.62 \ (\pm 0.13)</td>
<td>7.67 \ (\pm 0.13)</td>
</tr>
<tr>
<td>$pH_{max}$ [\text{-}]</td>
<td>9.04 \ (\pm 0.02)</td>
<td>9.034 \ (\pm 0.02)</td>
<td>9.04 \ (\pm 0.02)</td>
<td>9.03 \ (\pm 0.02)</td>
</tr>
<tr>
<td>$MIC$ [ppm]</td>
<td>145.53 \ (\pm 2.03)</td>
<td>149.84 \ (\pm 2.58)</td>
<td>145.52 \ (\pm 2.03)</td>
<td>152.08 \ (\pm 7.99)</td>
</tr>
<tr>
<td>$\mu_{opt}$ [h^{-1}]</td>
<td>2.21 \ (\pm 0.04)</td>
<td>2.18 \ (\pm 0.03)</td>
<td>2.21 \ (\pm 0.03)</td>
<td>2.20 \ (\pm 0.13)</td>
</tr>
<tr>
<td>$\beta$ [-]</td>
<td>-</td>
<td>12.44 \ (\pm 3.99)</td>
<td>-</td>
<td>1.33 \ (\pm 1.31)</td>
</tr>
<tr>
<td>MSE</td>
<td>0.284</td>
<td>0.276</td>
<td>0.283</td>
<td>0.268</td>
</tr>
</tbody>
</table>

As it can be seen, the interaction factor $\gamma(pH, [HAc])$ implemented by the model of Le Marc et al. [2] practically did not improve the MSE value obtained with the gamma model. This result was already expected since this interaction factor only has as an effect at extremely stressful conditions, where it is more aimed at predicting the growth/no-growth boundary than describing interactions for the growth rate. Indeed, for all the experimental conditions, the interaction factor was equal to 1.0.

The MSE value obtained with the model of Augustin and Carlier [1] presented a very small decrease of 3%. The value of $\beta$ gives an indication on the extent of the interactions. The high value estimated for this parameter (12.44) practically reduces the model of Augustin and Carlier [1] to the gamma equation without interactions, reason why the MSE value obtained is insignificantly lower and is insufficient to prove the existence of interactions between pH and $[HAc]$. The model of Augustin and Carlier [1] also includes the information on the extent of interactions on the growth limits, reason why the $pH_{min}$ obtained was lower than for the non-interactive gamma model and the model of Le Marc et al. [2], where interactions were found to be negligible. However, due to the lack of “no-growth” data, the growth/no-growth boundary is an extrapolation considering the present experimental data.

With the gamma-interaction model of Akkermans et al. [3], the MSE was reduced in 6%, comparing to the non-synergistic gamma model. As shown in Figure 6, for the effect of pH, $\gamma(pH, [HAc])$ is indeed very close to one for most the considered conditions and that, if any interactions are present, these are very little except when working at very low pH values. In the superoptimal pH region, gamma also seems to reach values clearly below 1.0 for high organic acid concentrations, but there is no data to support statements about interactions at this level. Although the gamma-interaction model (slightly) improves the MSE, it is highly questionable whether this improvement is significant or not. Assuming that interactions exist where, in fact, these are not present might cause growth boundaries to be estimated incorrectly and, therefore, result in unsafe food products. The fact that the gamma hypothesis could not be clearly contradicted given the current dataset does not mean, however, that for combinations of pH and $[HAc]$ different than those that were tested, interactions between these factors cannot occur in a more pronounced way.

Figure 6: Interaction factor $\gamma([-]$ of the gamma-interaction model of Akkermans et al. [3] as a function of pH [-] and $[HAc]$ [ppm].
4. Conclusions and future perspectives

The aim of this case study was to demonstrate the need for models that describe the combined effect of pH and acetic acid concentration on the maximum specific growth rate of *Escherichia coli*. An emphasis was given on the validity of the gamma concept, which assumes that environmental factors act independently of each other.

The relatively large amounts of acetic acid in the growth media of experiments in which this was not added had a great influence on the results, specifically on determining the separate effects of pH in the suboptimal range. Removing the acetic acid effect by dividing the experimental growth rates by the gamma factor for the correspondent undissociated acetic acid concentration, gave an indication of the separate pH effect. These results showed that if only the effect of pH was present, the growth rates for lower pH values would be higher, resulting in an even more extended plateau. Everything considered, in this case study, the individual effects of pH could not be clearly determined. This also demonstrates the relevance of accurately determining the amount of organic acid in the broth for each experiment, something that is not considered in many studies on this subject.

The separate effect of undissociated acetic acid concentration was studied by keeping pH constant. Considering the set of experiments at pH 6.50, a consistent decrease in the microbial growth rate was observed for increasing [HAc] values. The fact that the MIC was lower at pH 5.00 than at pH 5.50, 6.00 and 6.50 could mean that there is, in fact, an interaction between pH and acetic acid for more stressful conditions. However, it was observed that the MSE consistently decreased for lower pH values. It is very likely that the HPLC measurements present a low accuracy at lower pH conditions, meaning that the growth rates could have been estimated at [HAc] values different than those that were considered.

Given the current dataset, the gamma hypothesis could not be contradicted. In case interactions between pH and acetic acid exist, these are very limited as the largest decrease in the MSE value obtained with the interaction models was just 5%, correspondent to the gamma-interaction model of Akkermans et al. [3]. This improvement is highly questionable and, therefore, future research on interactions between pH and organic acids is required. This should focus on lower pH values and higher undissociated acid concentrations, where the gamma factors of both pH and acetic acid concentration are clearly below one. The most difficult problem to overcome is the fact that the gamma factor for pH is close to one for most conditions that allow microbial growth to occur and that growth might be hardly supported under those conditions, making the process of data collection more difficult. If the existence of such interactions can be proved, calibration on new data could be performed to investigate if the model could also be applicable for other *E. coli* strains, particularly pathogenic strains. The aim is, then, to develop an accurate model that can be applied to real food products, but to that end, its structure needs to be increased in complexity.

7. 2011 Outbreak of Rare *E. coli* Strain was Costly for Europe, in Food Safety News. 2011.