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

Rogério Pedro Brito Guerreiro

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Supervisors: Prof. Dr. Ir. J. F. Van Impe  
Prof. José António Leonardo dos Santos

**Examination Committee**  
Chairperson: Prof. Helena Maria Rodrigues Vasconcelos Pinheiro  
Supervisor: Prof. José António Leonardo dos Santos  
Member of the Committee: Dr. Carla da Conceição Caramujo Rocha de Carvalho

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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 relatively 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*
Resumo

Recentemente, a indústria alimentar tem procurado assegurar a segurança alimentar combinando diversos tratamentos / condições de armazenamento. Desta forma, existe um interesse acrescido em desenvolver modelos matemáticos que descrevam com exactidão a resposta dos microrganismos à combinação de diversos fatores ambientais. Estes fatores tanto podem apresentar uma ação independente, como interagirem entre si em condições de stress, produzindo um efeito inibitório superior ao esperado.

Neste estudo, é modelada a influência do pH e do ácido acético na taxa de crescimento de *Escherichia coli* K12. Com esta finalidade, foi efetuado um conjunto de experiências em biorreatores controlados por computador, em que as condições ambientais foram mantidas constantes. A estimativa de parâmetros foi aplicada diretamente às densidades celulares medidas, utilizando modelos para o pH e ácido acético retirados da literatura. As estruturas mais adequadas foram combinadas num modelo não-sinérgico, cuja validade foi testada por comparação com os modelos de Augustin and Carlier [1], Le Marc et al. [2] e Akkermans et al. [3].

A presença de quantidades relativamente elevadas de ácido acético no meio, a pHs mais baixos, resultou em menores taxas de crescimento, pelo que não foi possível isolar o efeito do pH. A reduzida melhoria na qualidade de ajuste conferida pelo modelo de Akkermans et al. [3] não permite comprovar a existência de interações entre os fatores estudados. Prevê-se a ocorrência de interações mais pronunciadas a valores de pH ainda mais reduzidos e concentrações de ácido acético mais elevadas, para as quais não havia dados experimentais que o comprovem.

**Keywords:** Taxa de crescimento; ácidos orgânicos; microbiologia predivita; biorreator; *Escherichia coli*
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Symbols and Abbreviations

\([\text{Ac}]_{\text{added}}\): Total concentration of acetic acid added to the growth medium

\([\text{Ac}]_{\text{BHI}}\): Total concentration of acetic acid present in the BHI

\([\text{Ac}]_{\text{measured}}\): Total concentration of acetic acid measured by HPLC

\([\text{HA}]\): Undissociated organic acid concentration

\([\text{HAc}]\): Undissociated acetic acid concentration

\([\text{OA}]\): Total concentration of organic acid

\(A\): Absorbance measured in the spectrophotometer

\(A_r\): Accuracy factor

\(AR\): Acid resistance

\(A_{\text{ref}}\): Reference value of the absorbance measured in the spectrophotometer

\(\text{ASP}\): Acid Shock Proteins

\(\text{ATR}\): Acid-tolerance response

\(a_{w, \text{max}}\): Theoretical maximum water activity for growth

\(a_{w, \text{min}}\): Theoretical minimum water activity for growth

\(a_w\): Water activity

\(B_f\): Bias factor

\(\text{BHI}\): Brain heart infusion broth

\(\text{BHIA}\): Brain heart infusion broth supplemented with agar

\(\text{cAMP}\): Cyclic adenosine monophosphate

\(\text{CDC}\): Centers for Disease Control and Prevention

\(\text{CGSC}\): Coli Genetic Stock Center

\(\text{CRP}\): cyclic adenosine monophosphate receptor protein

\(\text{Ec-NhaA}\): Sodium/proton antiporter of Escherichia coli
**EMP**: Embden-Meyerhof-Parnas pathway

**F**: Fisher information matrix

**g**: Generation time

**G**: Number of growing cells in the growing compartment at time $t$

**G_0**: Initial population capable of growth

**GRAS**: Generally Regarded as Safe

**HACCP**: Hazard Analysis Critical Control Point

**HPLC**: High-Performance Liquid Chromatography

**HUS**: Hemolytic-uremic syndrome

**J**: Jacobian matrix

**k**: Structural parameter related to the extent of optimum pH region for growth

**MIC**: Minimum inhibitory concentration

**MIC_{new}**: Minimum inhibitory concentration in the presence of interactions

**MRA**: Microbial Risk Assessment

**MSE**: Mean sum of squared errors

**N(t)**: Cell concentration at time $t$

**n(t)**: Natural logarithm of cell concentration at time $t$

**N_0**: Initial cell concentration

**n_0**: Natural logarithm of the initial cell concentration

**N_{max}**: Maximum cell concentration

**n_{max}**: Natural logarithm of the maximum cell concentration

**N_p**: Total number of model parameters

**N_i**: Total number of experimental data

**p**: Vapour pressure of water in the substrate (chapter 2) / set of model parameters (chapter 3)

**p^0**: Initial guess for the model parameters
\( p_0 \): Vapour pressure of the water at the same temperature as the food substrate

\( pHi \): Intracellular pH

\( pH_{\text{max}} \): Theoretical maximum pH for growth

\( pH_{\text{min, new}} \): Theoretical minimum pH for growth in the presence of interactions

\( pH_{\text{min}} \): Theoretical minimum pH for growth

\( pH_{\text{opt}} \): Theoretical optimum pH for growth

\( pKa \): Acidic dissociation constant

\( q(t) \): Natural logarithm of the physiological state of cells at time \( t \)

\( Q(t) \): Physiological state of cells at time \( t \)

\( q_0 \): Natural logarithm of the physiological state of cells

\( s_{pi}^2 \): Parameter variance

\( SSE \): Sum of squared errors

\( SSE_{\text{global}} \): Sum of squared errors for the full dataset

\( STEC \): Shiga toxin-producing \( Escherichia \) coli

\( T \): Temperature

\( t_a \): Time taken by cells for physiological changes and development of metabolic machinery

\( T_c \): Change Temperature

\( TCA \): Tricarboxylic Acid

\( t_m \): Time required for the metabolic machinery to produce energy and synthesize all biological components involved in cell replication

\( T_{\text{max}} \): Theoretical maximum temperature for growth

\( t_{\text{max}} \): Time at which the maximum cell concentration is reached

\( T_{\text{min}} \): Theoretical minimum temperature for growth

\( T_{\text{opt}} \): Theoretical optimum temperature for growth

\( V \): Parameter covariance matrix

\( V_{\text{inoc}} \): Inoculation volume
\( V_{\text{ref}} \): Inoculation volume of reference

\( \alpha \): Significance level (chapter 3) / Structural parameter (chapter 4)

\( \beta \): Structural parameter related to the extent of the interactions between environmental factors

\( \gamma \): Gamma factor for the growth rate reduction caused by a specific constraint

\( \eta \): Structural parameter related to the pH region for growth

\( \lambda \): Lag time

\( \mu \): Microbial specific growth rate

\( \mu_{\text{max}} \): Maximum specific growth rate
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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].

Although most *Escherichia coli* (*E. coli*) strains are harmless, others such as Shiga toxin-producing *Escherichia coli* (STEC) have been recognized as important food pathogens for decades [5]. The mildest symptoms of a STEC infection are, generally, stomach cramps, diarrhoea and vomiting. However, the potent toxins produced by this pathogenic strain (Shiga-like toxins) can also lead to fatal complications. According to the Centers for Disease Control and Prevention (CDC), 5-10% of the diagnosed STEC infections progress to the hemolytic-uremic syndrome (HUS), which can cause renal failure. Most of the reported cases and outbreaks of human illness have been attributed to the serotype O157:H7 [6]. Outbreaks of foodborne pathogens can have huge human and economic impacts. In 2011, an outbreak of STEC O104:H4 affected 3,950 people in Europe, causing death to 53 HUS patients. Fenugreek seeds imported from Egypt ended up being identified as the transport vehicle for this pathogen. However, an initial suspicion on Spanish cucumbers led to the destruction of tens of thousands of kilos of these vegetables and to losses of around 200 million euros a week [7].

There is, therefore, a great concern in quantifying 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). More concrete applications reside in the definition of critical control points within a Hazard Analysis Critical Control Point (HACCP) approach, shelf-life studies [8] or in the Microbial Risk Assessment (MRA) within food processing.

Compatible with the recent tendency in the food industry towards minimal food processing is the use of organic acids (e.g., acetic acid, lactic acid and citric acid). These 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. Among these, acetic acid is a popular food preservative 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. 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 [9-11]. At more stressful conditions, however, it is possible that synergistic interactions
between factors produce a combined effect which is greater than expected. Identifying and taking these interactions into account leads to more accurate model predictions [1-3].
2. Literature review

2.1. Food preservation

Food preservation can be defined as the action of maintaining the desired properties or nature of foods so that it remains safe and pleasant at the time of consumption [12]. Physical stress can be applied to microorganisms to induce cell death or to minimize microbial growth in food products. The main factors used for microbial growth prevention and, therefore, achieve food safety are temperature, pH and water activity. Even at low concentrations, organic acids (e.g., acetic acid, lactic acid and citric acid) are also often used in the food industry due to their preservative effect towards a broad spectrum of pathogenic and spoilage microorganisms. Other intrinsic (moisture content, oxidation-reduction potential, physical structure of the food, nutrients availability and presence of antimicrobial agents) and extrinsic (relative humidity, carbon dioxide and oxygen levels) factors can also contribute to the preservation of foods [13]. Whereas the influence of temperature [14, 15] and water activity [16] on the viability and growth of microorganisms has been reported in various previous studies, the mode of action of factors such as pH and organic acids is still not fully understood and their effects remain insufficiently quantified.

Cells can respond to stressful conditions either in an active or passive way. An active response is generally of the metabolic type. It requires energy consumption and the synthesis of intracellular molecules, such as heat shock or acid shock proteins (at high temperature or low pH conditions, respectively) and osmoregulatory solutes (for stressful water activity values). A passive response is a physical response that depends on both the gradient and the rate of physical perturbation between the intra- and the extra-cellular environment (e.g., water or solutes transfers through the membrane or a phase transition of the lipids of the membrane). Differences between the kinetics of the stress and the kinetics of the cell response may induce irreversible damages. Rapid cooling processes and high rates of pH reduction can lead to severe cell injuries or even death, whereas an intermediate cooling rate and a low rate of pH reduction may lead to an optimization of the cell survival through the induction of metabolic adaptive mechanisms [13].

Heat treatments and drying processes are widely used in the food industry to inhibit growth and destroy spores that may germinate and spoil food products. These are examples of traditional preservation processes (Figure 1). Traditional preservation processes are safe, economical, well established and have a broad application field. However, they provoke changes in the chemical, physical and nutritional properties of foods as their efficiency relies on the intensity and time of application of a single environmental factor [17].
Understanding the interactions between the different environmental factors allows the development of combined methods to achieve food stability and safety. This concept is behind the so-called “hurdle theory” [18]. In this approach, each growth-limiting factor (e.g., low pH, low water activity and low temperature) is referred to as a hurdle. Combining different hurdles may have an additive or, under specific conditions, a synergistic effect. In the absence of interactions between hurdles, these are combined in a multiplicative manner to produce an overall microbial growth inhibition. In case synergistic interactions between two environmental factors exist, these result in a combined effect that is greater than that expected from the simple multiplication of the individual factors [19]. The identification and quantification of these interactions can then be useful to select the constraints that best achieve an overall level of protection to food products with a minimal detrimental impact on the organoleptic properties of food products. Due to the milder preservation processes applied, hurdle technologies are more likely to meet consumers demand for “fresh”, safe, healthy and quickly prepared food [20].

2.1.1. Temperature

Temperature is one of the environmental factors with most known effects over the microbial growth rate, which has been extensively used in microbiology studies [3, 21, 22] and applied in the food industry to guarantee food safety [4] due to the impact it has on practically all reactions that occur within a cell [23]. The traditional preservation processes that act based on the inhibitory effect of
temperature are mainly sterilization, pasteurization, cooking, freezing and cooling processes. These processes may alter taste, texture and color properties of the food and, for heating processes, promote the digestibility of certain components and the coagulation of proteins [17].

A shift to high temperature values can cause the denaturation of essential enzymes and proteins, besides affecting the cell membrane, RNA, DNA and ribosomes. A “heat shock response” typically includes membrane permeability, intracellular concentration of solutes and the up regulation of heat shock proteins. Heat shock proteins have similar functions to those of chaperones, promoting the correct folding of new proteins or the refold of damaged proteins [13]. On the other hand, when exposed to temperatures lower than their optimum for growth, cells respond by suppressing heat shock proteins and inducing the synthesis of cold shock proteins (“cold shock response”). Cold shock proteins are produced during the adaptation to the new temperature conditions and assure energy generation through metabolic reactions, DNA supercoiling, RNA stability and proteins stability. To maintain the components on the cell membrane functional, the proportion of unsaturated fatty acids is increased during the adaptation to the environment to help maintain the fluidity of the membrane (homeoviscous adaptation) [23, 24].

### 2.1.2. Water Activity

Water is a basic element of food products. However, it is also responsible for a higher rate of undesired chemical/biochemical reactions. The existence of free water affects food stability and the organoleptic and nutritional properties of foods. Water activity ($a_w$) is a thermodynamic property that can be described as the relative equilibrium humidity of a product, i.e., its partial pressure of water vapour on the surface. It is a measure of the water available for chemical/biochemical reactions and is calculated by the ratio of the vapour pressure of water in the substrate (above the food) ($p$) and the vapour pressure of pure water at the same temperature ($p_0$) (equation 1) [12].

$$a_w = \frac{p}{p_0}$$

A food product is stable in terms of lipid oxidation, non-enzymatic browning and enzyme activity for $a_w$ values between 0.2 and 0.3. Lower water activity favours growth and metabolism perturbations, reducing the kinetics of spoilage reactions. Once a minimum water activity value for growth is reached, microbial growth is no longer possible and the spoilage reaction stops. This is the concept behind traditional preservation processes such as drying. During a drying process, the concentration of solutes in the liquid phase of the food increases as the water activity decreases, until the liquid phase is no longer present. At this point, the remaining water molecules have a strong interaction with the solid non-soluble compounds of food. The water molecules that do not participate in
hydrolysis reactions and escape from food as vapour are the ones that are tightly bound to water soluble compounds (e.g., sugar and salt gums) through osmotic binding or to the substrate matrix by a surface effect [12, 17].

The passive response mechanism of cells to low water activity levels is characterized by water and solute diffusion through the membrane, causing cell volume variation. The active microbial response is based on active osmoregulation systems that allow the accumulation of compatible solutes and, therefore, a control over the cellular volume and turgor pressure. Microbial response to shifts in water activity can also be related to the use of humectants that modify the amount of water available for biochemical reactions [25].

2.1.3. pH

Low pH is an extensively used constraint in food-manufacturing processes such as pickling or fermentation to prevent microbial growth and ensure food stability and food safety. However, pathogenic E. coli strains have already revealed to be tolerant to low pH. For this reason, it is important to understand the microbial response to different pH values and accurately determine the risk associated to the different food products [9].

In Table 1 and Table 2 are given typical pH values for plant-origin and animal-origin food products, respectively. Fruits such as pineapple, raspberries or strawberries have low pH values, which are inhibitory for most bacteria. On the other hand, meats, raw milk and most milk derived products have pH values above 5.6, making them more sensitive to bacterial growth and consequently to food spoilage. Vegetables have their characteristic pH closer to that of animal-origin foods and are, therefore, more prone to contamination than most plant-origin food products. The susceptibility of a food product to contamination is defined by the correspondent pH range for growth [26].

Typically, the pH values for growth and survival of bacteria are in the range 4.00-8.00, whilst yeasts and molds can grow in a broader range of pH values [27]. For this reason, yeasts and molds are often associated with spoilage of pickled, acidified, and fruit products [23].
Table 1: Characteristic pH values of plant-origin food products [26].

<table>
<thead>
<tr>
<th>Food</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peas (canned)</td>
<td>5.71-6.00</td>
</tr>
<tr>
<td>Pineapple</td>
<td>3.20-3.64</td>
</tr>
<tr>
<td>Pineapple (canned)</td>
<td>3.39-3.59</td>
</tr>
<tr>
<td>Plums (Blue and Damson)</td>
<td>2.78-2.89</td>
</tr>
<tr>
<td>Plums (red and yellow)</td>
<td>3.62-4.95</td>
</tr>
<tr>
<td>Potatoes</td>
<td>5.40-5.90</td>
</tr>
<tr>
<td>Quince (stewed)</td>
<td>3.12-3.37</td>
</tr>
<tr>
<td>Raspberries</td>
<td>3.62-3.95</td>
</tr>
<tr>
<td>Rhubarb (stewed)</td>
<td>3.24-3.34</td>
</tr>
<tr>
<td>Spaghetti (cooked)</td>
<td>5.97-6.40</td>
</tr>
<tr>
<td>Spinach (cooked)</td>
<td>6.60-7.18</td>
</tr>
<tr>
<td>Strawberries</td>
<td>3.30-3.35</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>3.99-4.75</td>
</tr>
<tr>
<td>Tomatoes (canned)</td>
<td>4.10-4.25</td>
</tr>
<tr>
<td>Vegetables soup (canned)</td>
<td>5.16</td>
</tr>
<tr>
<td>Vinegar</td>
<td>3.12</td>
</tr>
<tr>
<td>Watermelon</td>
<td>5.18-5.60</td>
</tr>
<tr>
<td>Worcestershire sauce</td>
<td>3.83-3.87</td>
</tr>
<tr>
<td>Yeast</td>
<td>5.66</td>
</tr>
</tbody>
</table>

Table 2: Characteristic pH values of animal-origin food products [26].

<table>
<thead>
<tr>
<th>Foods</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef (broth)</td>
<td>6.14-6.20</td>
</tr>
<tr>
<td>Beef (ground)</td>
<td>5.10-6.20</td>
</tr>
<tr>
<td>Beef (raw)</td>
<td>5.60</td>
</tr>
<tr>
<td>Buttermilk</td>
<td>4.41-4.83</td>
</tr>
<tr>
<td>Butter</td>
<td>6.10-6.40</td>
</tr>
<tr>
<td>Cheese (camembert)</td>
<td>7.44</td>
</tr>
<tr>
<td>Cheese (cheddar)</td>
<td>5.90</td>
</tr>
<tr>
<td>Cheese (cottage)</td>
<td>4.75-5.02</td>
</tr>
<tr>
<td>Cheese (roquefort)</td>
<td>5.41-6.10</td>
</tr>
<tr>
<td>Chicken</td>
<td>6.20-6.40</td>
</tr>
<tr>
<td>Cream</td>
<td>6.40-6.60</td>
</tr>
<tr>
<td>Eggs (whole)</td>
<td>6.58</td>
</tr>
<tr>
<td>Eggs (white)</td>
<td>7.96</td>
</tr>
<tr>
<td>Eggs (yolk)</td>
<td>6.10</td>
</tr>
<tr>
<td>Lobster</td>
<td>7.10-7.43</td>
</tr>
<tr>
<td>Milk</td>
<td>6.30-6.50</td>
</tr>
<tr>
<td>Oysters</td>
<td>5.68-6.17</td>
</tr>
<tr>
<td>Sardines</td>
<td>5.42-5.93</td>
</tr>
<tr>
<td>Shrimp</td>
<td>6.80-7.00</td>
</tr>
<tr>
<td>Soda crackers</td>
<td>5.65-7.32</td>
</tr>
<tr>
<td>Tuna (canned)</td>
<td>5.92-6.10</td>
</tr>
</tbody>
</table>

In Table 3 are shown the optimum pH range for growth and the internal pH values for various microorganisms. Generally, organisms with an optimal pH for growth between 0.50 and 5.00 are called acidophiles, whereas neutrophils and alkaliphiles grow best in the range 5.00-9.00 and 9.00-12.00, respectively. The pH range for growth can also be influenced by the competing microflora, damage caused by prior heat treatments or water activity [23, 28].
Table 3: pH range for growth and characteristic internal pH values for various microorganisms [28].

<table>
<thead>
<tr>
<th>Organism</th>
<th>pH for growth</th>
<th>Internal pH (pHi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcylobacillus</td>
<td>2.0 to 5.0</td>
<td>5.9 to 6.1</td>
</tr>
<tr>
<td>Acetobacterium</td>
<td>2.8 to 4.3</td>
<td>4.0 to 6.0</td>
</tr>
<tr>
<td>C. thermoaceti</td>
<td>5.0 to 8.0</td>
<td>5.7 to 7.3</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>2.35 to 8.6</td>
<td>6.0 to 7.3</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>4.4 to 9.1</td>
<td>7.2 to 7.4</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>4.4 to 8.7</td>
<td>7.5 to 8.2</td>
</tr>
</tbody>
</table>

Taken from Booth and Kroll (1989)

The decrease in the external pH in an acidification process is not as efficient in controlling microbial growth as changes in the intracellular pH (pHi) but can also lead to a loss of viability. Strong acids are known to lower the external pH and denature cell surface (outer and inner membrane, cell wall, periplasm) enzymes. Structures such as flagella, pili, chemoreceptors and other periplasmic proteins are highly susceptible to damage caused by changes in the external pH. When adaptation is not possible, functions such as flagellar motility are lost, meaning that cells cannot move to environments where nutrient, pH or chemical conditions are more favourable [29]. Bacterial cells at acidic pH conditions have also been found to be more sensitive to metal ion toxicity (Cu^{2+}, Cd^{2+} or Zn^{2+}, e.g.), reason why tolerance mechanisms might include outward-directed metal ion pumps and the repression of uptake systems for these ions and of detoxification mechanisms. In alkaline conditions, these ions are a lot less available. However, some of them are essential for growth and only alkaliphiles are thought to have uptake systems capable of retrieving these metals under these conditions [30].

Unlike weak lipophilic acids, strong acids are incapable of permeating through the cytoplasmic membrane. Instead, their accumulation in the extracellular environment creates a pH gradient that increases membrane permeability to protons. By diffusing into the cytoplasm, protons reduce the intracellular pH, interfering with the synthesis of essential components and other metabolic functions [28]. Booth and Kroll [28], in 1989, have also reported a diminished activity of ion transport systems, responsible for depriving the cell from up taking ions that are essential for growth.

To keep pHi in the optimum range of values (pH homeostasis), bacteria use a combination of passive and active mechanisms. Passive mechanisms are based on the selective permeability property of the cell membrane, which prevents protons from entering the cytoplasmic environment. In this way, in case the external pH varies, microbial cells are capable of avoiding large changes in the pH. Cells exposed to acidic environments can also increase their level of proteins and glutamates in the cytoplasm and their buffering capacity [23]. This buffering capacity is conferred by the acid and base side chains of the proteins and by the phosphate groups of nucleic acids [26]. Active homeostasis is based on potassium ion and proton movement. Unlike strong acids, weak organic acids penetrate
the membrane in a protonated form and overcome the passive homeostasis mechanisms. To preserve metabolic functions, it is then necessary to force protons back to the extracellular environment. This creates a membrane potential which prevents further proton removal. To disrupt this membrane potential, potassium ions and other cations diffuse to the intracellular environment without affecting the pH\textsubscript{i} value as this depends exclusively on the hydrogen ion concentration. A transmembrane pH gradient is created and the internal pH is kept close to optimum values for growth. This mechanism, however, can be compromised at low extracellular pH values [23, 28].

Another response mechanism involved in *E. coli* cells resistance to acidic stress is thought to involve increased proportions of saturated and cyclopropane fatty acids, with the latter being produced when growth is close to ceasing and at the cost of high amounts of energy [31]. Both types of fatty acids have been suggested to decrease the membrane permeability to protons by increasing its rigidity at low pH conditions [23, 32, 33].

Adaptation to alkaline environments has not been as extensively studied as acid pH homeostasis. Even so, it is known that a shift to high pH values induces stress on bacteria such as *E. coli*, provoking a response with SOS (a DNA repair mechanism) and heat shock-like proteins [34, 35]. One of the possible consequences of the alkalisation of the medium is a small increase in the pH\textsubscript{i}, sufficient to make intracellular protons less available for enzymes, affecting their activity [36]. Involved in the alkaline pH homeostasis are (i) the metabolic synthesis of acid by-products by amino acid deaminases and sugar fermentation, (ii) enhanced activity of the ATP synthase, which transports protons to the cytoplasm and generates ATP, (iii) modification of cell surface characteristics and, above all, (iv) monovalent cation/proton antiporters [37]. Besides the potassium/proton antiporter, *E. coli* is known to have at least three more cation/proton antiporters. There is strong evidence on the relevance of a sodium/proton antiporter (NhaA) for alkaline pH homeostasis [38]. This antiporter must be electrogenic rather than electroneutral, ensuring that the number of protons entering the cell is higher than the number of sodium cations transferred to the extracellular environment so that the net positive charge moves from outside the cell to the cytoplasm. Thus, a membrane potential is created to promote proton movement to the intracellular environment and, consequently, the acidification of the cytoplasm (Figure 2). For pH values higher than 9, the sodium/proton antiporter of *E. coli* (Ec-NhaA) is thought to be unable to capture extracellular protons [37]. The transport of small extracellular substances through outer porin proteins is also thought to enhance cell resistance to high extracellular pH values [36].

Applying large pH gradients might damage food sensorial properties. Therefore, acidification processes should be used in combination with other preservation methods, e.g., in a synergistic action with weak lipophilic organic acids [26].
Figure 2: Role of the sodium/proton antiporter of *Escherichia coli* (Ec-NhaA) in maintaining pH homeostasis in alkaline environments. One sodium cation is exchanged for two protons to create a membrane potential and promote proton inward movement [39].

### 2.1.4. Organic acids

Organic acids have long been combined with other preservation factors due to their antimicrobial activity. These are weak lipophilic acids that can be added to beverages, vinegar, pickled vegetables, mayonnaise, salad dressings and other food products to prevent or slow down the growth of pathogenic and spoilage microorganisms. Their structure differs in the number of carboxyl (from one to three) and hydroxyl groups, in the number of carbon-carbon double bonds and in its aromatic or aliphatic character [40]. In a study with pickle brines, acetic acid treatment has been found to be more effective than low pH alone in decreasing the viability of *E. coli* O157:H7 [41].

Both the undissociated (protonated) and dissociated forms of an acid contribute to microbial growth inhibition. However, the mode of action of organic acids is mainly linked to the capacity of their undissociated form to freely diffuse across lipid bilayers and reduce the cytoplasmic pH. In a first step, low extra-cellular pH values favour the undissociated form of organic acids, which can permeate the cell membrane (Figure 3, A). Once it reaches the cytoplasm, the undissociated organic acids face a higher pH due to cell buffers and dissociation occurs. Protons are released and the intracellular pH (pHₙ) decreases to values incompatible with growth as essential metabolic reactions are inhibited (Figure 3, B) [23].
Figure 3: Growth inhibition mechanism of weak organic acids [23].

For this reason, the effectiveness of an acid in inhibiting microbial growth is a function of the ratio of undissociated to dissociated acid at a given environmental pH [42]. The amount of undissociated organic acid ([HA]) can be determined from its total concentration ([OA]) by the Henderson-Hasselbalch equation (equation 2) [9].

$$[HA] = \frac{[OA]}{1 + 10^{pH - pK_a}}$$

$pK_a$ is the acidic dissociation constant, i.e., the pH at which the concentrations of the undissociated and dissociated forms of an acid are equal. Most effective compounds have acidic $pK_a$ values and exist mainly in the dissociated form at neutral pH values [43]. Unlike organic acids, strong acids (e.g., HCl) tend to readily dissociate in aqueous solutions or normal food products. For this reason, they do not cross the cell membrane and the cytoplasmic pH remains unaltered [42].

Organic acids are also known to chelate elements which are essential for growth, e.g., iron [9]. Other possible effects of their antimicrobial action include membrane disruption [44] and accumulation of toxic ions [45].

Yeast are known to respond to the weak acid stress by removing the hydrogen ions in excess using proton pumps on the plasma membrane (H+-ATPase), which requires energy from the hydrolysis of ATP molecules (Figure 3, C). The resistance mechanisms of bacteria are related to the acid-tolerance response (ATR) and acid-resistance (AR) systems. Different ATR systems can be induced
in different phases of microbial growth. The exponential-phase (see section 2.3.1) ATR system is based on the expression of, at least, 50 acid shock proteins (ASPs). Similarly to the heat shock proteins, ASPs have the function of protecting or repairing macromolecules from the damage caused by the low pH conditions. The synthesis of 8 of these proteins is regulated by the alternative sigma factor RpoS. In the stationary-phase (see section 2.3.1) ATR system, only 15 ASPs are expressed, 4 of which are also expressed during the exponential phase [23].

There are four distinct AR systems in *Escherichia coli*, which are induced at extremely low pH conditions. Acid resistance system 1 (AR1) is the oxidative or glucose-repressed system and occurs in bacteria that can oxidize organic compounds as part of their metabolism. Although its structural components and mechanism are not clearly established, it is known that this system is induced during the stationary phase of growth, where the alternative sigma factor RpoS is involved in the regulation of cell physiology. The cyclic adenosine monophosphate (cAMP) receptor protein CRP is also involved in the expression of this system and is known to regulate the function of ion channels [46]. This system can be induced during the exponential phase of growth or at the beginning of the stationary phase. AR2 and AR3 are the glutamate and arginine-dependent systems, respectively. AR2 uses the decarboxylation of glutamate to consume intracellular protons and, therefore, maintain pH homeostasis. AR3 only differs from AR2 in the sense that it also requires the decarboxylation of arginine. For these systems to act it is then necessary that both amino acids are present in the medium. At last, AR4 is based on the action of the lysine decarboxylase [47].

Although ATR and AR mechanisms act primarily in protecting cells from low pH stress, these have also been reported by Baik et al. [48] to enhance the response to the loss of viability caused by weak organic acids. In turn, Rhee et al. [49] gave evidence on the existence of antagonistic effects between acetic acid and pH for specific combinations of these factors, since a concentration of 0.5% of this weak acid in mustard was found to be less inhibitory of *E. coli* O157:H7 growth than pH alone. Bjornsdottir et al. [50] have also shown that the acetic acid, among other organic acids, can protect *E. coli* cells from low pH stress when present in concentrations of 5 to 10 mM.

Interactions between organic acids and other preservation factors have also been reported. The inhibitory effect of organic acids was shown to increase with the incubation temperature [51] and decrease with the presence of salt, which is thought to enhance the AR response mechanisms of several microorganisms [42].

**Acetic acid and acetate metabolism by *Escherichia coli***. Acetic acid (Figure 4) is a monocarboxylic acid used as a food preservative in commercial mayonnaise, dressing and sauces manufacturing processes and is present in vinegar in concentrations of 5% to 20% [52]. According to the United States Food & Drug Administration [53], this weak lipophilic acid is generally regarded as safe (GRAS) for diverse applications and general-purpose usage. Along with lactic acid, it has been
reported as one of the most efficient organic acids in inhibiting the growth of spoilage microorganisms [23].

**Figure 4:** Chemical structure of the acetic acid [54].

The production of acetic acid in aerobically growing *E. coli* occurs when the uptake of primary carbon source (e.g., glucose) is higher than its conversion to CO₂ and biomass. At high glucose concentrations, there is a downregulation of the glucose uptake rate by the phosphotransferase system. A membrane-bound glucose-specific protein, Enz II<sub>glc</sub>, is believed not to respond to the normal regulation signals and an extra flux of glucose enters the central metabolic pathways to produce more acetyl-CoA than required for biosynthesis and energy generation. A portion of this flux can, however, enter the Embden-Meyerhof-Parnas (EMP) pathway and the resulting additional pyruvate is combined with coenzyme A to yield acetyl-CoA. One acetyl-CoA is converted to acetate by orthophosphate acetyltransferase and acetate kinase, yielding one ATP molecule [55].

Acetate is particularly consumed when glucose is at limiting values. First, it is converted back to acetyl-CoA. Then, acetyl-CoA is oxidized through the Tricarboxylic Acid (TCA) cycle and the electron transport chain. In this way, some of the glucose destined to energy metabolism is spared for biosynthetic use [55].

### 2.2. Predictive microbiology

The emergence of foodborne microbial pathogens of small size, high reproduction rate, phenotypic plasticity and genetic adaptation created the need to accumulate large amounts of quantitative information on microbial behaviour in food products. This, however, requires an extensive amount of work and is costly [56].

Predictive microbiology is a rapid and costly-effective alternative to traditional testing, where mathematical models are built to describe microbial growth responses to the different environmental conditions experienced in foods. Applications of these models include shelf-life studies, identification of critical points within a Hazard Analysis Critical Control Point (HACCP) approach [57]. With the increased relevance of milder food processing as a mean to achieve food safety without damaging
the organoleptic properties of foods, predictive microbiology has also been dedicated in assessing the combined effects of environmental conditions on microbial growth [42]. Environmental factors can be independent of each other over a wide range of conditions and produce an additive effect when combined [10, 58]. Other view states that interactions might occur for more stressful combinations of these factors, particularly near the growth/no-growth boundaries, resulting in a combined effect which is less than expected [1-3].

Models can be generically classified as descriptive and explanatory models. Descriptive models are empirical, i.e., experimental data is required to perform parameter estimations. This is usually a trial-and-error process, where parameters are estimated for various structures and by comparing the results. Examples of models of this class are the polynomial models, artificial neural networks and principal component analysis. Explanatory (mechanistic) models give a scientific meaning, or, at least, measurable physiological processes to the collected data. Models can also be either deterministic or stochastic. Deterministic models are used to predict the state of the system at any point in time by combining known initial conditions and a mathematical function which describes the time evolution of the system. Models that study the probability distributions of data are called stochastic or probabilistic models [59].

**2.2.1. Primary models**

Primary models are used in predictive microbiology to describe the number of cells as a function of time, i.e., the microbial growth curve (Figure 5). These can be generally classified as growth or survival models. However, only the first are of the scope of this research. Bacterial growth curves are generally of the sigmoid type on a semi-logarithmic plot, where the natural logarithm of cell concentration is plotted over time [59].

![Figure 5: Microbial growth curve of E. coli K12 growing at a constant temperature of 35°C](image)

**Figure 5:** Microbial growth curve of *E. coli* K12 growing at a constant temperature of 35°C [60].
The growth of a microbial population is essentially described by three consecutive stages: the lag phase, the exponential phase and the stationary phase. The lag phase corresponds to the introduction of microorganisms in fresh culture medium, e.g., from processing equipment or human skin onto foods, with an initial cell concentration $N_0$ [CFU/mL]. Cell division does not occur during this phase as bacteria are adapting to the new environmental conditions, depleted of ATP, essential cofactors and ribosomes required for growth. New enzymes are also synthesized so that the new nutrients can be consumed. Meanwhile, cells begin to replicate their DNA and to increase in mass as a preparation for cell division [61]. The lag time ($\lambda$ [h]) depends mainly on the type and phenotype of the bacteria, the inoculum size and the level of stress applied to the cell culture. In a growth curve, the lag time corresponds to intersection of the tangents to the lag and exponential phases [62].

At the exponential phase, microbial population has uniform chemical and physiological properties and cell division occurs at a constant rate which is at its maximum value ($\mu_{\text{max}}$ [h$^{-1}$]). The microbial specific growth rate ($\mu$ [h$^{-1}$]) corresponds to the number of cell divisions per unit of time (equation 3) and can be related to the generation time ($g$ [h]) by equation 4. $N(t)$ [CFU/mL] is the cell concentration (number of microorganisms per unit volume) at time $t$ [h] [63].

$$\mu = \frac{1}{N} \cdot \frac{\partial N}{\partial t} \quad (3)$$

$$g = \ln(2) \cdot \frac{1}{\mu} \quad (4)$$

Exponential growth occurs until an essential nutrient is depleted from the medium or the accumulation of a toxic metabolite produced by cells reaches inhibitory levels. At this point, bacterial growth slows down until it ceases and the so-called stationary phase begins. This final stage of growth is described by the maximum cell concentration, $N_{\text{max}}$ [CFU/mL], which is in truth an asymptote, as microbial populations can get very close to that value but never reach it. To obtain the sigmoid curve of Figure 5 and for computational purposes, $N(t)$ and the other parameters with respect to cell concentration are generally replaced by its natural logarithm $n(t)$ [ln(CFU/mL)] [64].

### 2.2.1.1. The Logistic and Gompertz models

The modified logistic (equation 5) [65, 66] and Gompertz (equation 6) [67-69] models are used to relate the natural logarithm of the relative cell density ($y = \ln(N(t)/N_0)$) with time $t$. The original equations contained mathematical parameters ($a$, $b$, $c$, ...) with no biological significance, which makes it difficult to find adequate initial values and to calculate confidence intervals of parameters that are not estimated directly on the equation. For this reason, the original models were...
reparameterized and the mathematical parameters were replaced by \( \mu_{\text{max}}, \lambda \) (as previously defined) and the asymptotic level of the growth curve \( A = \ln\left( \frac{N_{\text{max}}}{N_0} \right) \), \( e \) is the Euler number [70].

\[
y = \frac{A}{1 + \exp\left\{ \frac{\mu_{\text{max}}(\lambda-t)+2}{A} \right\}} \quad (5)
\]

\[
y = A \cdot \exp\left\{ -\exp\left[ \frac{\mu_{\text{max}}e}{A} (\lambda - t) + 1 \right] \right\} \quad (6)
\]

The modified logistic function has been used, among other applications, to model fish spoilage [71] and colony diameter of fungi [72]. Even so, it has not been as extensively used as the modified Gompertz model, which is asymmetric around the inflection point and, therefore, offers a better description of the growth curve as it is. The modified Gompertz function has long been used to model the growth of microorganisms such as Bacillus cereus [73] or Listeria monocytogenes in skim milk medium [74]. Other applications of this model include modelling the growth of spoilage bacteria in beer [75], fresh-cut vegetables [76] and raw ground beef [77]. However, it is also known to underestimate the generation time up to 13% and to predict negative lag times for certain data sets, requiring too many data points to cover the whole growth range [59].

### 2.2.1.2. Baranyi and Roberts model

The previous models are practical but not quite mechanically or kinetically acceptable. Baranyi and Roberts [78] proposed, in 1994, an extended non-autonomous version of the logistic function that imposes a time dependence on microbial growth. The motivation behind the development of this model is the recovery of microbial growth in static batch cultures, with controlled environmental conditions. This consists of a set of differential equations (equations 7 and 8) based on the pure exponential growth (equation 3), with the cell density \( N(t) \) [CFU/mL] being a function of time \( t \). \( N_{\text{max}} \) [CFU/mL] and \( \mu_{\text{max}} \) [h\(^{-1}\)] are as previously defined [59]. \( Q(t) \) [-] is a measure of the physiological state of cells and is proportional to the concentration of a limiting substrate [59, 78].

\[
\frac{dN(t)}{dt} = \frac{Q(t)}{1+Q(t)} \cdot \mu_{\text{max}} \cdot \left( 1 - \frac{N(t)}{N_{\text{max}}} \right) \cdot N(t) \quad (7)
\]

\[
\frac{dQ(t)}{dt} = \mu_{\text{max}} \cdot Q(t) \quad (8)
\]
$1 - (N(t)/N_{max})$ is a logistic type inhibition function that describes the stationary phase. During exponential growth, the number of cells increases towards a maximum value. Therefore, the inhibition function is a monotonically decreasing function which assumes values between one and zero. This function is purely empirical and lacks cause-effect relationships, i.e., does not include the exhaustion of essential substrates or accumulation of toxic metabolites. For this reason, the model is not suitable for more complex and realistic situations such as co-culture growth. $\alpha(t) = Q(t)/(1 + Q(t))$ is the adjustment function and is used to describe the lag phase of the growth curve. It is monotonically increasing function, with values between (approximately) zero and one, and is mechanistically inspired. For computational purposes, a natural logarithmic transformation can be applied to the model equations, resulting in [60, 78]:

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

(9)

$$\frac{dq(t)}{dt} = \mu_{max}$$

(10)

$n(t)$ [ln(CFU/mL)] and $q(t)$ [-] are the natural logarithms of $N(t)$ and $Q(t)$, respectively. $n_0$ and $q_0$ are the correspondent initial values. This model is particularly advantageous when applied to dynamic experiments, since it is available as a set of differential equations, but for static environmental conditions it also presents good fitting capacity and has biologically interpretable parameters, which facilitates the establishment of cause-effect relationships. For these reasons, it is widely accepted for primary modelling in predictive microbiology studies [60]. Applications of this model include the growth of several microorganisms including *E. coli* [79], colony diameter of heat-resistant fungi [80] and spoilage in green asparagus [81] and salads [82].

### 2.2.1.3. Buchanan three-phase linear model

A simpler three-phase linear model was developed and compared to the Baranyi and Gompertz models by Buchanan et al. [25], in 1997. This model admits that the three phases of bacterial growth are described as straight lines on semi-logarithmic plots. The lag phase is represented by a flat segment where $\mu$ is precisely zero (equation 11), whereas the exponential phase is an inclined segment with the exponential growth rate as slope (equation 12). Finally, the stationary phase is also described as a straight line at the level of the natural logarithm of the maximum population density (equation 13). $n(t)$ and the model parameters $n_0$, $n_{max}$ and $\lambda$ are as previously defined. $t_{max}$ is the
time at which \( n(t) = n_{\text{max}} \). A characteristic three-phase linear plot and the four parameters of the model are represented in Figure 6.

\[
\text{Lag phase: } n(t) = n_0, \quad t \leq \lambda
\]  \hspace{1cm} (11)

\[
\text{Exponential phase: } n(t) = n_0 + \mu_{\text{max}}(t - \lambda), \quad \lambda < t < t_{\text{max}}
\]  \hspace{1cm} (12)

\[
\text{Stationary phase: } n(t) = n_{\text{max}}, \quad t \geq t_{\text{max}}
\]  \hspace{1cm} (13)

Figure 6: Typical growth behavior as described by the three-phase linear model [25].

In this model, the lag time is assumed to result from the sum of two distinct periods (equation 14). \( t_a \) (equation 16) is the time taken by the cells for physiological changes and development of metabolic machinery. \( t_m \) (equation 15) is the time required for the metabolic machinery to produce energy and synthesize all the biological components involved in cell replication. The latter is constant and equivalent to the duplication time as all daughter cells are admitted to produce energy and new cellular components at the same rate [25].

\[
\lambda = t_a + t_m
\]  \hspace{1cm} (14)

\[
t_m = g
\]  \hspace{1cm} (15)

\[
t_a = \lambda - g
\]  \hspace{1cm} (16)
This model has been used by its authors to predict *E. coli* O157:H7 growth behavior, with the results showing that it is more "robust" than the modified Gompertz [67-69] and Baranyi and Roberts models [78]. However, its quality-of-fit is only comparable to that of the other models in cases where the variance associated to the microbial population is small [25].

### 2.2.1.4. McKellar model

Back in 1997, McKellar [83] proposed a simplified version of the Baranyi and Roberts model [78], using the same parameters. It is, however, a compartmental model, since it makes a distinction between growing and non-growing populations and assumes that growth occurs from a small portion of cells from the growing compartment at $t = 0$. All the subsequent growth follows a logistic-type equation (equation 17). $G$ is the number of growing cells in the growing compartment at time $t$. $G_0$ is the initial population capable of growth. $\mu_{max}$ and $N_{max}$ are as previously defined [59].

$$\frac{dG(t)}{dt} = G(t) \cdot \mu_{max} \cdot \left(1 - \frac{G(t)}{N_{max}}\right)$$

(17)

This model has proved to present a better fit to growth data than the Gompertz equation [67-69]. However, in comparison with the model of Baranyi and Roberts [78], this model has limited applications due to its compartmental assumptions that make it not adequate for most of non-linear regression programs [59].

### 2.2.2. Secondary models

Secondary models act as an extension of the primary models by describing the effect of environmental conditions on growth dynamics, particularly on the maximum specific growth rate (hereafter also referred to as growth rate). These can be compared based in their (i) simplicity, (ii) biological significance of the parameters, (iii) minimum number of parameters, (iv) applicability, (v) quality-of-fit, (vi) minimum structural correlations and (v) ease of initial parameter estimation [84].

Secondary model parameters can be estimated either with a *one-step* or a *two-step method*. According to the one-step method, primary models (microbial growth rate as a function of time) are combined with the secondary models (microbial growth rate as function of the environmental conditions) so that parameter estimation is performed directly on the measured cell concentrations. On the other hand, the two-step method is performed by fitting a secondary model on the growth
rates estimated using a primary model. The latter method is more extensively used in food microbiology, but objective function of the one-step method is more in line with the aim of food microbiology, i.e., the minimization or prevention of microbial growth in foods for a given period. Furthermore, the two-step method places to much confidence in data with high variance in comparison with the one-step method [85].

There are essentially two groups of secondary models. The first group includes the square root models, the cardinal parameter models and the Arrhenius-based models. The second group refers to neural networks and response surface models. The models that compose the first group are mainly characterized for having biologically or graphically interpretable parameters and a high fitting capacity. The models of the second group are known to require no a priori knowledge of the relationship between the model inputs and outputs, as well of its model structure. In this way, they are highly flexible in comparison with the models of the first group [24].

The need for predictive models that accurately describe the growth at stressful conditions of different preservation factors has led to an extensive use of square root and cardinal parameters models. Square root-type models use a square root transformation to stabilize the variance [22]. In comparison, cardinal parameter models have the advantage of containing only biologically interpretable parameters that can be used to predict theoretical growth limits and optimal conditions [84]. The most attractive properties of these models are the reduced number of required parameters, the biological interpretability of those parameters and the possibility of being easily used in sequential modelling approaches in combined effect studies [3].

In this section, the main square root and cardinal parameter models used for the individual and combined effects of environmental factors are discussed. A special focus on the effects of pH and undissociated organic acid concentration on the microbial growth rate is given.

2.2.2.1. Temperature effect

Temperature is one of the major environmental factors influencing microbial growth in foods. The most common secondary models for the separate effect of temperature are of the Arrhenius, cardinal parameter and square root types. The first have long been used by microbiologists in bacterial growth studies and state that the relationship between the growth rate and the inverse of temperature is linear for the whole biokinetic temperature range [86]. This assumption, however, has been shown by Ratkowsky et al. [22] not to correspond to the behavior presented by microorganisms, resulting in a poor fit to data. Furthermore, this type of models does not comply with progressive modelling approaches, where the individual effects of temperature and other environmental conditions must be clearly identifiable from the combined effect [3, 59].
**Square root (SQRT) models.** The role that refrigeration processes have in the prevention of pathogenic and spoilage bacteria growth makes it relevant to estimate growth rates at the suboptimal temperature range. It is also within this range that temperature during storage and distribution varies. The suboptimal temperature range is characteristic of each microorganism [87]. The relationship between $\mu_{max}$ [h$^{-1}$] and temperature $T$ [°C] in the suboptimal range has been reported to follow the model proposed by Ratkowsky [22] in 1982 (equation 18), and is valid for many bacteria. The observed linearity results from a square root transformation of the experimental growth rates. $T_{min}$ [°C], the theoretical minimum temperature for growth, and $b$, a constant, are the model parameters.

$$\sqrt{\mu_{max}(T)} = b \cdot (T - T_{min})$$

(18)

Generally, equation 18 can be used to model the effect of temperature on microbial growth until optimum conditions, $T_{opt}$ [°C], are reached. For temperatures higher than $T_{opt}$, the decrease in $\mu_{max}$ can be described by an extended version of the Ratkowsky model for the suboptimal temperature region [22]. In his extended model [88] (equation 19), a parameter for the superoptimal temperature range is introduced: $T_{max}$ [°C], which represents the theoretical maximum temperature for growth. Beyond this temperature, growth ceases, i.e., the growth rate is assumed to be zero. These parameters only give an indication of the growth limits, which means growth can occur outside this region. It is, therefore, important that predictions are made as accurately as possible. Van Derlinden et al. [24] have applied this model to the growth of *E. coli* K12 and observed the existence of a "change temperature", $T_c$ [°C], in the suboptimal temperature region, below which the decrease in the growth rate is lower than expected. A similar behavior had already been reported for *L. monocytogenes* [2, 89]. The existence of this change temperature implies that the $\sqrt{\mu_{max}(T)} - T$ relationship is only linear in the range $[T_{min}, T_c]$ and is most likely linked to metabolic changes caused by the cold shock response [24].

$$\sqrt{\mu_{max}(T)} = b \cdot (T - T_{min}) \cdot (1 - \exp(c \cdot (T - T_{max})))$$

(19)
Figure 7: Effect of temperature on the maximum specific growth rate of E. coli K12 MG1655 with the square root model (SQRT) of equation 19. \( T_{\text{min}} = 7.75 \, ^\circ\text{C} \); \( T_{\text{max}} = 47.4 \, ^\circ\text{C} \) [24].

CTMI. The Cardinal Temperature Model with Inflection (CTMI, equation 20) [84] was empirically built to describe the separate effect of temperature on the microbial growth rate without mechanistic explanations. The advantages of this model are the use of exclusively interpretable parameters and the inclusion of three cardinal temperatures \( (T_{\text{min}}, T_{\text{opt}}, T_{\text{max}}) \) and \( \mu_{\text{opt}} \) [h\(^{-1}\)], the optimum growth rate, reached at \( T_{\text{opt}} \).

\[
\mu_{\text{max}}(T) = \begin{cases} 
0, & T \leq T_{\text{min}} \\
\frac{(T-T_{\text{min}})(T-T_{\text{max}})}{(T_{\text{opt}}-T_{\text{min}})(T_{\text{opt}}-T_{\text{max}})(T_{\text{opt}}-T_{\text{min}}-2T)}, & T_{\text{min}} < T < T_{\text{max}} \\
0, & T \geq T_{\text{max}}
\end{cases}
\] (20)

It is also implicit in the model definition that the growth rate is equal to zero for temperatures below and above \( T_{\text{min}} \) and \( T_{\text{max}} \), respectively. The ratio \( \mu_{\text{max}}(T)/\mu_{\text{opt}} \) expresses the reduction of the growth rate caused by a non-optimal temperature [59].

The CTMI has been adapted by other authors to account for deviations from the microbial response described by this model. Le Marc et al. [2] introduced an adaptation of the CTMI (aCTMI) based on the particular behavior presented by \( L.\ monocytogenes \) at the suboptimal temperature range [2, 89]. This was done by adding two more parameters to the original model structure: the change temperature \( T_c \) [°C] and \( T_1 \) [°C]. \( T_1 \) corresponds to the temperature at which the equation for temperatures above \( T_c \) and below \( T_{\text{opt}} \) is equal to zero. Adapting the CTMI model to these deviations at suboptimal temperatures resulted in an improved accuracy, with higher predicted growth rates in this region, since linearity is exclusively verified for temperatures lower than \( T_c \) [2, 3].
The aCTMI, however, possesses two more parameters and presents a discontinuity that may cause errors during numerical computations. Recently, Akkermans et al. [3] has proposed the bCTMI as a continuously differentiable adaptation of the CTMI. These model parameters are the same as the CTMI and produce lower $\mu_{max}$ estimates than the CTMI. A graphical comparison of the three cardinal temperature models is presented in Figure 8.

![Figure 8: Growth rate estimates by the CTMI (—) and its adaptations: aCTMI (---) and bCTMI (—–) [3].](image)

2.2.2.2. Water activity effect

SQRT. McMeekin et al. [90] studied the combined effect of temperature and water activity on the growth rate of *Staphylococcus xylosus*, a halotolerant microorganism. To describe the growth rate reduction caused by the separate effect of water activity, the authors used equation 21, where $a_{w, min}$ [-] is the parameter referent to the theoretical minimum water activity that sustains growth and $c$ a proportionality constant. As it is clear from this model structure, it is assumed that $\mu_{max}(a_w)$ is directly proportional to the difference between $a_w$ and $a_{w, min}$ [90].

$$\sqrt{\mu_{max}(a_w)} = c \cdot \sqrt{a_w - a_{w, min}} \quad (21)$$

Similarly to temperature, an extended version of the previous square root model was proposed by Miles et al. [91] to cover the full biokinetic range of water activity values. In the model of Miles et al. [91] (equation 22), a parameter for theoretical maximum water activity, $a_{w, max}$ [-], is included in the structure. $C$ and $d$ are proportionality constants determined by parameter estimation. This model has shown a good quality of fit to *Vibrio parahaemolyticus* [91] and *E. coli* [63] growth data.
\[ \sqrt{\mu_{\text{max}}(a_w)} = C \cdot \sqrt{(a_w - a_{w\text{ min}}) \cdot (1 - \exp(d \cdot (a_w - a_{w\text{ max}})))} \] (22)

**Cardinal Model of Rosso and Robinson [92].** A cardinal model for the effect of water activity was proposed by Rosso and Robinson [92] in a study involving the growth of moulds. This model structure is equal to that of the CTMI (equation 20), but with \( a_{w\text{ min}} \), \( a_{w\text{ opt}} \), \( a_{w\text{ max}} \) replacing the cardinal temperatures.

Humectants are known to modify water activity according to a linear relationship [25]. Models expressing the microbial growth rate as a function of solute concentration instead of \( a_w \) have also been used in predictive microbiology studies [20].

### 2.2.2.3. pH effect

**SQRT.** Wijtzes et al. [93] proposed two secondary models to describe the effect of stressful pH conditions on the reduction of the microbial growth rate. In an analogous way to temperature and water activity effects, it is assumed that growth limits are defined by a theoretical minimum (\( pH_{\text{min}} \)) and maximum (\( pH_{\text{max}} \)) pH for growth. One of the models proposed by the authors considers a parabolic \( \mu_{\text{max}}(pH) - pH \) relationship (equation 23). The square root is omitted for simplicity. \( d_1 \) is a regression coefficient [93].

\[ \mu_{\text{max}}(pH) = -d_1 \cdot (pH - pH_{\text{min}}) \cdot (pH - pH_{\text{max}}) \] (23)

A different description for the effect of pH in the suboptimal and superoptimal regions is given by the same authors in equation 24 [93], where the factor for high pH values involves an exponential function. This model is generally preferred over the previous one in cases where the optimum pH for growth is far from being precisely in the middle of \( pH_{\text{min}} \) and \( pH_{\text{max}} \). In every other case, equation 23 should be used over equation 24, since it has two parameters without biological meaning, \( d_2 \) and \( d_3 \).

\[ \mu_{\text{max}}(pH) = -d_2 \cdot (pH - pH_{\text{min}}) \cdot [1 - \exp(d_3 \cdot (pH - pH_{\text{max}}))] \] (24)

Baranyi and Roberts [78] reported that microbial growth rate is described by a plateau in the surroundings of the optimum pH and by a sudden decrease as pH is reduced to growth inhibiting values. Cole et al. [21] added that this decrease of \( \mu_{\text{max}} \) is proportional to the increase of protons \([H^+]\) \([\text{mM}]\). Based on this observations and in the definition of pH, Presser et al. [9] developed a pure pH effect model (equation 25), where \( pH_{\text{min}} \) is as previously defined and corresponds to the maximum hydrogen ion concentration that allows growth. \( f \) is another constant of proportionality. In
the plot of Figure 9 it is possible to observe that the growth rate goes from an asymptote towards a threshold value as pH decreases.

\[ \mu_{max}(pH) = f \cdot (1 - 10^{pH_{min} - pH}) \]  

(25)

Figure 9: pH [-] influence on the microbial growth rate [1/min] calculated by the model of Presser et al. [9]. The lines correspond to the model predictions for different lactic acid concentrations. The optimum pH for growth was determined to be 7 [9].

Cardinal pH Model (CPM) [84]. Rosso et al. [84] developed a Cardinal pH Model (CPM, equation 26) similar to the CTMI to describe the influence of pH on the microbial growth rate, using four cardinal parameters: \( pH_{min} [-] \), \( pH_{max} [-] \), the optimum pH for growth \( pH_{opt} [-] \) and \( \mu_{opt} \) [h\(^{-1}\)]. \( \mu_{opt} \) is the optimum growth rate, i.e., the maximum specific growth rate reach at optimum pH conditions \( (pH_{opt}) \). This model structure was built over the same assumptions of the previous cardinal parameter models, i.e., containing only biologically interpretable parameters and not admitting the occurrence of growth outside the growth boundaries. It is also symmetric around \( pH_{opt} \), with a similar response to changes in the suboptimal and superoptimal pH ranges. However, between pH 6.5 and 8.5, \( E. \ coli \) has the capacity to maintain pH homeostasis, i.e., to avoid large changes in the cytoplasmic pH despite the variations of the extracellular pH [94]. This ability results from a combination of passive and active mechanisms, among which the existence of intracellular buffers [23, 26] and is not taken into account by the CPM.
The existence of an extended plateau in the optimum pH range has been recently admitted by an adaptation of the Cardinal pH Model, the srCPM (equation 27), proposed by Akkermans et al. [3]. This flattened optimum is obtained by raising the model structure of the CPM to $1/k$. $k$ is a shape parameter larger than or equal to 1. The value of $k$ can be determined by parameter estimation or by fixing it to the value that best describes the relationship between $\mu_{\text{max}}$ and pH, which avoids introducing an additional parameter (devoid of biological significance) in the model structure. In cases where $k$ is set equal to 2, the resulting model structure corresponds to the square root of the CPM. Higher $k$ values typically correspond to a more flattened optimum (Figure 10) [3].

$$
\mu_{\text{max}}(\text{pH}) = \begin{cases} 
0, & \text{pH} \leq \text{pH}_{\text{min}} \\
\mu_{\text{opt}} \cdot \frac{ \text{pH} - \text{pH}_{\text{min}} \cdot \text{pH} - \text{pH}_{\text{max}} }{ (\text{pH} - \text{pH}_{\text{min}} \cdot \text{pH} - \text{pH}_{\text{max}}) - (\text{pH} - \mu_{\text{opt}})^2 }, & \text{pH}_{\text{min}} < \text{pH} < \text{pH}_{\text{max}} \\
0, & \text{pH} \geq \text{pH}_{\text{max}} 
\end{cases}
$$

(26)

Figure 10: The effect of the shape parameter $k$ on the microbial growth rates estimated by the srCPM. The model output is represented for $k$ equal to 1 (— — — —), 2 (— — — —), 3 (— — — —) and 4 (— — — —) [3].

The previous pH models assume a similar microbial growth responses for the suboptimal and superoptimal pH ranges. However, the way acid and alkaline stress affect cells and the pH homeostasis mechanisms involved can be structurally different. To account for this difference in the suboptimal and superoptimal pH ranges, Akkermans et al. [3] proposed the cardinal parameter model aCPM [3].
aCPM (equation 28). This structural modification of the original CPM model [84] was done by raising to the power $\eta$ the pH factors for the suboptimal range in both numerator and denominator and also includes the shape parameter $k$ for the flattened optimum referent to the pH homeostasis mechanisms. The effect of the structural parameter $\eta$ in the model output is presented in Figure 11 [3].

$$
\mu_{max}(pH) = \begin{cases} 
0, & pH \leq pH_{min} \\
\mu_{opt} \cdot \left(\frac{(pH-pH_{min})^\eta(pH-pH_{max})}{(pH-pH_{min})^\eta(pH-pH_{max})-(pH-pH_{opt})^2}\right)^{1/k}, & pH_{min} < pH < pH_{max} \\
0, & pH \geq pH_{max}
\end{cases}
$$

(28)

Figure 11: The effect of $\eta$ on the microbial growth rates estimated by the aCPM. The model output is represented for $k$ equal to 3 and $\eta$ equal to 1 (---), 2 (--), 3 (---) and 4 (....) [3].

The outputs of the three cardinal pH models mentioned as function of the environmental pH can be observed in Figure 12 [3].
Figure 12: Model outputs of the different cardinal parameter models for the effect of pH on the microbial growth rate: aCPM (---), srCPM (---) and CPM (---) [3].

2.2.2.4. Undissociated organic acid effect

The growth inhibitory effect of organic acids has been reported to be mostly influenced by the concentration of the undissociated form, although dissociated organic acids also contribute to a growth rate reduction. The inhibitory potential of these acids is related to their theoretical minimum inhibitory concentration $MIC$ [ppm], which corresponds to the smallest amount of (undissociated) organic acid capable of preventing microbial growth and is also influenced by the external pH. Presser et al. [9] developed a model for the undissociated organic acid effect assuming that $\mu_{\text{max}}$ varies linearly with the difference between the $MIC$, constant for a given bacterial strain, and the concentration of the undissociated form of the acid $[HA]$ [ppm] (equation 29). $[HA]$ can be obtained from the total concentration of the acid by applying the Henderson-Hasselbalch equation (equation 2). The model structure corresponds to a square root model where the square root is omitted and is illustrated in Figure 13 [9].

$$\mu_{\text{max}}([HA]) = \mu_{\text{opt}} \cdot (1 - \frac{[HA]}{MIC})$$ (29)
Figure 13: Dependence of the microbial growth rate [1/min] of *E. coli* M23 on the concentration of undissociated lactic acid (mM) for total acid concentrations of 25 (squares), 50 (circles) and 100 (triangles) mM. The observed line was obtained by linear regression [9].

When studying the effects of acetic acid, an organic acid known to have food preservation properties [53], on the $\mu_{\text{max}}$ of *Listeria innocua*, Le Marc et al. [2] observed that these were better described by applying a square root to term for the undissociated acid concentration (equation 30).

$$\mu_{\text{max}}([HA]) = \mu_{\text{opt}} \cdot (1 - \frac{[HA]}{MIC})$$

(30)

pH and undissociated acid concentration are two widely used hurdles in the food industry whose definitions are closely related as discussed in sections 2.2.3 and 2.2.4. To know if their combination produces a multiplicative or a synergistic effect on microbial growth prevention is of considerable interest for more accurate model predictions and to develop milder food processes involving these two factors. In the absence of interactions, the overall level of protection results from multiplying the separate effects of both hurdles (gamma hypothesis) [63, 90, 95]. Synergistic effects present a greater growth-inhibitory potential and may be described by the inclusion of interaction factors [1-3].

### 2.2.2.5. Gamma hypothesis

According to the gamma hypothesis, different environmental factors act independently on the inhibition of microbial growth. The growth rate reduction caused by each hurdle $E_i$ is represented by a gamma factor ($\gamma_{E_i}(E_i)$). The structure for this nonsynergistic combined effect model is essentially
obtained from the multiplication of the gamma factors for all the environmental factors considered (equation 31) [63, 90, 95].

\[
\mu_{\text{max}}(e) = \mu_{\text{opt}} \cdot \gamma_{E_1}(E_1) \cdot \gamma_{E_2}(E_2) \cdot \gamma_{E_3}(E_3) \cdot \cdots \cdot \gamma_{E_n}(E_n)
\]  

(31)

The advantages of the gamma hypothesis are essentially the reduced work load and the possibility of describing the combined effect of different environmental conditions directly from the correspondent separate effects. However, combinations of certain environmental factors have proved to be more synergistic than independent, particularly at more stressful levels [1-3]. Synergistic combinations produce an inhibitory effect greater than the expected, resulting in lower growth rates than those predicted by the gamma model of equation 31. However, caution should be taken when assuming the existence of interactions, as this can lead to fail-safe predictions of the growth boundaries in case the gamma hypothesis is valid.

The gamma model has been revealed to describe sufficiently well the effects of pH and organic acids on the growth rate of different microorganisms by other authors [96]. However, studies as that of Rhee et al. [49] and Baik et al. [48] suggest that the microbial response to stressful combinations of pH and organic acids might be more complex than the described by the gamma model of equation 32.

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

(32)

2.2.2.6. 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. In this way, information obtained from the growth limits can be used for better growth rate predictions. The new suboptimal range parameters, \(pH_{\text{min,new}}\) [-] and \(MIC_{\text{new}}\) [ppm], are determined by equations 33 and 34, respectively [1]. \(\beta\) [-] is a shape parameter and, therefore, has no biological meaning. However, this can give an idea on the extent of the interactions. Lower values of \(\beta\) result in smaller growth regions and consequently also in lower growth rates and in more pronounced interactions, as shown by Akkermans et al. [3].

\[
pH_{\text{min,new}}([HA]) = pH_{\text{opt}} - (pH_{\text{opt}} - pH_{\text{min}})(1 - \frac{[HA]}{MIC})^{1/\beta}
\]  

(33)
\[ M\text{IC}_{\text{new}}(pH) = M\text{IC}. (1 - \left(\frac{pH_{\text{opt}} - pH}{pH_{\text{opt}} - pH_{\text{min}}}\right)^\beta) \] \tag{34}

A consequence of the way the new suboptimal parameters are calculated is that the gamma factors for pH and undissociated acid concentration become also dependent on the other environmental variable, as described in equation 35 \[1\].

\[ \mu_{\text{max}}(pH, [HA]) = \mu_{\text{opt}} \cdot \gamma(pH, [HA]) \cdot \gamma_{[HA]}([HA], pH) \] \tag{35}

### 2.2.2.7. Model of Le Marc et al.

The model of Le Marc et al. \[2\] describes the synergistic effects at the growth/no-growth limits by introducing a factor for interactions in the non-synergistic gamma model of equation 32. The original model included gamma factors for the effects of temperature, pH and organic acid concentration on the growth kinetics of \textit{Listeria innocua} \[95\]. For the case where only the interactions between pH and undissociated organic acid concentrations are considered, this assumes the form of equation 36.

\[ \mu_{\text{max}}(pH, [HA]) = \mu_{\text{opt}} \cdot \gamma(pH, [HA]) = \mu_{\text{opt}} \cdot \gamma_{pH}(pH, [HA]) \cdot \gamma_{[HA]}([HA], pH) \cdot \gamma_i(pH, [HA]) \] \tag{36}

The interaction factor \(\gamma_i(pH, [HA])\) is calculated assuming the existence of three distinct regions for combinations of environmental conditions (equations 37 to 40): (i) the environmental factors do not interact and the combined effect is the product of the separate effects \(\gamma_i(pH, [HA]) = 1\), (ii) synergy between the environmental factors causes a growth rate reduction greater than the one predicted from the independent effects \(\gamma_i(pH, [HA]) = 2 \cdot (1 - \xi(pH, [HA]))\), (iii) growth ceases due to the combination of constraints \(\gamma_i(pH, [HA]) = 0\). \(\delta\) is a threshold value fixed to 1/2 based on a set of theoretical assumptions by Le Marc et al. \[2\].

\[ \gamma_i(pH, [HA]) = \begin{cases} 
1, & \xi(pH, [HA]) \leq \delta \\
2 \cdot (1 - \xi(pH, [HA])), & \xi(pH, [HA]) < 1 \\
0, & \xi(pH, [HA]) \geq 1 
\end{cases} \] \tag{37}

\[ \xi(pH, [HA]) = \frac{1}{2} \left[ \frac{\varphi_{pH}(pH)}{1 - \varphi_{pH}(pH)} + \frac{\varphi_{[HA]}([HA])}{1 - \varphi_{[HA]}([HA])} \right] \] \tag{38}

\[ \varphi_{pH}(pH) = (1 - \gamma(pH))^2 \] \tag{39}
\[
\varphi_{[HA]}([HA]) = (1 - \gamma([HA]))^2
\]  

(40)

2.2.2.8. Gamma-interaction model of Akkermans et al.

Akkermans et al. [3] proposed a model structure (equation 41), referred to as gamma-interaction model by the author, where the interaction factors introduced include a shape parameter \( \beta \). Similarly to the structural parameter of the model of Augustin and Carlier [1], this does not have a clear biological meaning but can be related to the extent of interactions between environmental conditions. \( e \) is the set of studied environmental conditions and \( \gamma_i(e) \) is the interaction factor [3].

\[
\mu_{max}(e) = \mu_{opt} \cdot \gamma(e) = \mu_{opt} \cdot [\prod_{j \in e} \gamma_j(E_j)] \cdot \gamma_i(e)
\]  

(41)

The gamma-interaction model was originally applied by its author to the effects of temperature and pH on the growth rate of \( E. coli \) K12. However, its structure was built in a way that it can be applied to the combination of different environmental factors [3]. When applied to the specific case of pH and undissociated organic acid it can be written as:

\[
\mu_{max}(pH, [HA]) = \mu_{opt} \cdot \gamma(pH, [HA]) = \mu_{opt} \cdot \gamma_{pH}(pH) \cdot \gamma_{[HA]}([HA]) \cdot \gamma_i(pH, [HA])
\]  

(42)

\[
\gamma_i(pH, [HA]) = [1 - (1 - \gamma_{pH}(pH)) \cdot (1 - \gamma_{[HA]}([HA]))]^\beta
\]  

(43)

The closer \( \beta \) is to zero, the weaker are the interactions between the studied environmental conditions. Such as the Augustin and Carlier [1] and the Le Marc et al. [2] synergistic models, the model structure proposed by Akkermans et al. [3] was found to be applicable over a sequential modelling approach, as it allows the separate effects to be identified from the combined effect model. In fact, when interactions are absent, it is reduced to the gamma hypothesis (equation 32) by setting \( \beta \) to zero. Of all the three interaction models discussed, this model includes the parameter \( \beta \) that is more related to the existence and extent of synergies, even though it has no biological meaning. In contrast with the other combined effect models is the fact that different shape parameters can be used to model interactions between different sets of conditions [3].
3. Aim of the study

The current project was developed at BioTeC+: Chemical & Bioprocess Technology and Control, from KU Leuven, under the supervision of Professor Doctor Jan Van Impe. The context of this work is within Simen Akkerman’s PhD, whose goals are to develop quantitative description methods for microbial dynamics under the stress caused by multiple environmental conditions.

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 (a non-pathogenic strain) 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.
4. Materials and methods

4.1. 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). Table 4 shows the formula of the BHI broth from Oxoid/VWR and the concentration of each component.

Table 4: Formula of the BHI broth from Oxoid/VWR and the concentration of each component in grams per liter.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain infusion solids</td>
<td>12.5</td>
</tr>
<tr>
<td>Beef heart infusion solids</td>
<td>5.0</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>2.5</td>
</tr>
<tr>
<td>Final pH @ 25°C</td>
<td>7.4 (± 0.2)</td>
</tr>
</tbody>
</table>

4.2. Inoculum preparation

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 BHIA 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.
4.3. Inoculation of the bioreactor

This procedure was designed so that the bioreactor could be inoculated with a cell concentration around 7 ln(CFU/mL). This concentration value corresponds to the minimum measurable number of cells via the plate counting technique, which allows the cell culture to start as far from the maximum population density as possible. All the following steps were performed under sterile conditions.

First, 1 mL of preculture was transferred from the prepared inoculum to an Eppendorf. From this Eppendorf, 20 µL of inoculum was transferred to a falcon tube of 50 mL containing 15.980 mL of fresh BHI. To calculate the volume of inoculum to be transferred from the falcon tube into the bioreactor, optical density measurements of the diluted preculture were performed in a spectrophotometer (FilterMax F5), at a wavelength of 595 nm. The inoculation volume, $V_{inoc}$, was then determined from the average of the measured absorbance values, $A$, using equation 44. In this equation, $A_{ref}$ and $V_{ref}$ are the reference absorbance and volume values and are equal to 0.130 and to 4.00 mL, respectively. These reference values were chosen so that the inoculation volume would not be too small to be accurately transferred with a syringe into the bioreactor. However, different combinations of $A_{ref}$ and $V_{ref}$ for all experiments would have been possible as well. Finally, the correct inoculation volume was transferred from the falcon tube containing the preculture to a second 50 mL falcon tube. Using a syringe and a needle, the bioreactor was inoculated with the inoculum present in the second falcon tube, through the inoculation port.

$$V_{inoc} = \frac{V_{ref}A_{ref}}{A}$$

(44)

4.4. Bioreactor experiments

Experiments were performed in a set of computer controlled bioreactors (BioStat B, Sartorius Stedim 336 GmbH) operating in batch mode. The reactor vessels had a total volume of 5 L and were, in all cases, filled with 3 L of BHI broth. The amount of acetic acid (Table 5) added to the bioreactor depended on the experimental design (see section 3.5). The prepared BHI broth was then autoclaved following the manufacturers’ instructions.

Table 5: Experimental data on the acetic acid used for the bioreactor experiments [96].

<table>
<thead>
<tr>
<th>Acid</th>
<th>Chemical formula</th>
<th>Molecular Weight (MW) [g/mol]</th>
<th>Acid supplier</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>C₂H₄O₂</td>
<td>60.05</td>
<td>Acros Organics, New Jersey</td>
<td>4.75</td>
</tr>
</tbody>
</table>
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, glass membrane pH electrode (Hamilton Company). This electrode was calibrated before each experiment using pH 7 and 4 phosphate (K₂HPO₄) buffer solutions. After autoclaving the bioreactor, pH measurements were corrected for temperature by taking a sample (at the control temperature) and measuring the correspondent pH value on an external device. In case the difference between the externally measured pH and the pH value of the bioreactor was superior to 0.03, a re-calibration of the electrode as explained in the bioreactor manual was performed. For each experiment, pH was kept at a constant value by adding acid (0.5 M H₂SO₄, Sigma-Aldrich) or base (1 M KOH, Thermo Fisher Scientific) solutions through peristaltic pumps. A PID controller was used to steer the pumps.

Aeration was set to 0.2 L/min after autoclaving. The oxygen concentration was measured with a polarographic electrode (Ingold) and controlled at the stabilized level during the experiment. The vessel content was stirred at 75 rpm with Rushton impellers. 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). 1 mL of antifoaming agent (Y-30 emulsion, Sigma-Aldrich) was added prior to every experiment to prevent foaming.

4.5. 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.

4.6. Experimental design

An overview of the experimental design is represented in Figure 14. Initially, experiments were designed in a way that allowed the effects of pH and acetic acid concentration to be investigated.
individually, by keeping one environmental condition close to optimal and testing different values of
the other. 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 4 experiments performed at pH 6.50 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 14: pH [-] and total acetic acid concentration, [Ac] [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)

**4.7. Parameter estimation and confidence intervals**

The extensively used primary model of Baranyi and Roberts [78] was used to describe the microbial
population [ln(CFU/mL)] as a function of time [h]. 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:

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

(45)
In equation 45, \( n_{m,i}(t_i) \) 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 parameter estimation \( p_i \) were determined from the Student’s t-distribution and the parameter variance \( s^2_{p,i} \) (equation 46). The value for \( s^2_{p,i} \) was obtained from the main diagonal of the parameter covariance matrix \( V \) (equation 47). \( \alpha \) is the significance level and was equalled to 0.05. Being \( N_t \) the total number of experimental data and \( N_p \) the total number of parameters, \( N_t - N_p \) gives the number of degrees of freedom. Lower uncertainties are characteristic of more accurate parameter estimations.

\[
\left[ p_i \pm t_{\frac{\alpha}{2},N_t-N_p} \sqrt{s^2_{p,i}} \right] \quad (46)
\]

\[
s^2_{p,i} = V(i,i) \quad (47)
\]

The covariance matrix \( V \) can be approximated by the inverse of Fisher Information Matrix \( F \) [97] (equation 48).

\[
V = F^{-1} \quad (48)
\]

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 49. The MSE (equation 50) 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.

\[
F = \frac{1}{MSE} J^T J \quad (49)
\]

\[
MSE = \frac{SSE}{N_t-N_p} \quad (50)
\]

In the secondary modelling, the cardinal pH models CPM [84] (equation 26), srCPM [3] (equation 27) and aCPM [3] (equation 28) were compared by their description of the separate effect of pH on the growth rate of \textit{E. coli} K12. The separate effect of the undissociated form of the acetic acid was modelled using a similar model structure to those of Presser et al. [9] (equation 29) and Le Marc et al. [2] (equation 30). Combined effect modelling was performed based on the gamma model (equation 32) and the models of Augustin and Carlier [1] (equations 33 to 35), Le Marc et al. [2] (equations 36 to 40) and Akkermans et al. [3] (equations 42 and 43). Secondary model parameters were estimated.
by performing a one-step parameter estimation, i.e., directly on the cell density data by combining the primary model with the secondary models. In this way, the objective function of the one-step method is also the minimization of the error on the predicted microbial response, given by the sum of squared errors for the full dataset ($SSE_{global}$, equation 51). The quality-of-fit of the secondary models was also assessed by calculating the MSE of equation 50 with $SSE_{global}$.

$$SSE_{global} = \sum_{j=1}^{N_t} \sum_{i=1}^{N}(n_j(t_i) - n_j(t_i, p))^2 \tag{51}$$

The Jacobian matrix ($J$) was initially supplied to the *lsqnonlin* routine as an optimization options structure in the secondary modelling parameter estimation procedure. $J (N_t \times N_p)$ is formed by the partial derivatives of the model output $n(t)$ to the model parameters $p$ at each sampling time $t$, $\partial n(t)/ \partial p_i$, which reflect the sensitivity of the model output to (small) changes in the model parameters. This matrix was initially computed for an initial guess of the undetermined model parameters $p^0$. Each iteration, $p^0$ was replaced by the new parameter estimates until convergence was accomplished. The expressions for the sensitivity equations were obtained by integrating the following differential equation:

$$\frac{d}{dt} \left( \frac{\partial n(t)}{\partial p_i} \right) = \frac{\partial \mu_{max}}{\partial p_i} \cdot (1 - \exp(n(t) - n_{max}))) - \mu_{max} \cdot \exp(n(t) - n_{max}) \cdot \frac{\partial n(t)}{\partial p_i} \tag{52}$$

The sensitivity of the maximum specific growth rate $\mu_{max}$ to the secondary model parameters depends on the considered model structure and can be generically written as equation 53, where $E_i$ is the environmental factor influencing the growth rate. The calculus behind the sensitivity equations and the Jacobian matrix is explained in more detail in the Appendix.

$$\frac{\partial \mu_{max}(E_i)}{\partial p_i} = \mu_{opt} \cdot \frac{\partial \gamma(E_i)}{\partial p_i} \tag{53}$$

In the one-step parameter estimation, both primary and secondary model parameters are determined. Growth rates were computed and plotted against the growth rates estimated using the model of Baranyi and Roberts [78] just for a better comparison between secondary models.
Models were also assessed by their accuracy factor $A_f$ and bias factor $B_f$:

$$A_f = \exp\left(\sqrt{\frac{\sum_{i=1}^{N_t} (\ln(\mu_{p,i}(p)) - \ln(\mu_{m,i}))^2}{N_t}}\right)$$  \hspace{1cm} (54)$$

$$B_f = \exp\left(\frac{\sum_{i=1}^{N_t} (\ln(\mu_{p,i}(p)) - \ln(\mu_{m,i}))}{N_t}\right)$$  \hspace{1cm} (55)$$

$\mu_{m,i}$ is the “experimental” or “measured” growth rate and $\mu_{p,i}(p)$ the predicted growth rate for a set of parameters $p$. Although the objective of the one-step parameter estimation is the natural logarithm of cell density, $\ln(\mu_{max})$ is a valid approximation under many experimental conditions. Lower $A_f$ values correspond to more accurate model predictions. $B_f$ values above or below one correspond to overestimated or underestimated growth rates, respectively.
5. Results and discussion

5.1. Undissociated acetic acid in the growth medium

Table 6 shows the undissociated acetic acid concentrations \([HAc]\) for the 26 static experiments performed. These values were determined from the amounts of acid known to have been added to the bioreactor, \([Ac]_{added}\), and from the results of an HPLC analysis performed on samples taken prior to the beginning of each experiment, with the aim of quantifying the amount of acetic acid added as brain heart infusion solids (BHI), \([Ac]_{BHI}\). The Henderson-Hasselbalch equation (equation 2) was used to convert the total concentration of acetic acid, \([Ac]_{measured}\) [ppm], to the concentration of the undissociated form, which has been shown to have the most inhibitory effect. In experiments where \([Ac]_{BHI}\) was not measured, an average value for the BHI from the correspondent manufacturer was admitted. This was only done for experiments where the concentration of undissociated acid is very low, making this calculation acceptable.
Table 6: Total concentrations of acetic acid added to the growth medium, \([\text{Ac}]_{\text{added}}\) [ppm], present in the brain heart infusion solids (BHI), \([\text{Ac}]_{\text{BHI}}\) [ppm], and in the bioreactor at the beginning of the batch, \([\text{Ac}]_{\text{measured}}\) [ppm]. The Henderson-Hasselbalch equation (equation 2) was used to compute the concentration of undissociated acetic acid, \([\text{HAc}]\) [ppm], from \([\text{Ac}]_{\text{measured}}\) [ppm].

- Average concentration of acetic acid (dissociated and undissociated) in the BHI broth from Oxoid: a
- Average concentration of acetic acid (dissociated and undissociated) in the BHI broth from VWR: b

The HPLC measurements revealed the existence of relatively large amounts of acetic acid in the BHI broth, leading to high undissociated acetic acid concentrations in experiments performed at low pH conditions. In Figure 15, the pH-[\text{HAc}] conditions for the full set of experiments are plotted for a better comparison with the experimental design (Figure 14). The presence of these considerably high
amounts of the undissociated form is more relevant for experiments 1, 2 and 8. In these experiments, no acetic acid was added with the purpose of assessing the separate effect of pH. However, undissociated acetic acid was shown to be present in concentrations of 72.29 ppm, 49.13 ppm and 24.25 ppm, respectively. This fact must, then, be considered when discussing the growth of E. coli K12 at different pH values. In experiments performed at neutral and alkaline conditions, the environmental pH favoured the dissociation of the acetic acid molecules and, therefore, it is not expectable that the presence of acetic acid in the medium influences the microbial population growth, with pH being the only factor at stressful levels.

![Graph](image)

**Figure 15**: pH [-] and undissociated acetic acid concentration, $[\text{HAc}]$ [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).

### 5.2. Primary modelling

A primary model was used to describe E. coli K12 population as a function of time. The model of Baranyi and Roberts [78] (equations 9 and 10) was fitted to the cell density data derived from each set of conditions. This model was chosen due to its wide acceptability in predictive microbiology studies [25, 62, 94, 98], good fitting capacity and its biologically meaningful parameters. $\mu_{\text{max}}$ [h$^{-1}$] is the maximum specific growth rate and is obtained during the exponential phase. By following the routine described in section 3.7, $\mu_{\text{max}}$ can be estimated along with the natural logarithms of the initial cell concentration $n_0$ [ln(CFU/mL)], maximum cell concentration [ln(CFU/mL)] and physiological state of cells at time $t = 0$, $q_0$ [-].
Equation 56 [78] was used to relate \(q_0\) to the lag time \(\lambda\) [h], since its values are easier to interpret from the biological and graphical points of view.

\[
q_0 = \ln\left(\frac{1}{\exp(\lambda \cdot \mu_{\text{max}}) - 1}\right)
\]  

(56)

In all experiments, temperature was kept at an optimum value of 37°C [99]. In this way, the only environmental conditions studied were pH and/or undissociated acetic acid concentration, according to the values shown in Table 6. This research focuses in modelling the maximum specific growth rate. To estimate the maximum specific growth rate as accurately as possible, a large number of data points was retrieved from the exponential phase of the growth curve. As all parameters are estimated simultaneously, the uncertainty on \(n_0\), \(q_0\) and \(\lambda\) also influences the error on \(\mu_{\text{max}}\). Therefore, cell density data was also obtained from the lag and stationary phases of growth.

In primary modelling, \(\mu_{\text{max}}\) is also dependent on the environmental conditions, but these were just kept constant for every experiment. For this reason, the effects of pH and undissociated acetic acid on the microbial response can be assessed by plotting and comparing the growth curves.

5.2.1. pH effect on microbial growth

Figure 16 shows the natural logarithm of cell density measurements (triangles), \(n(t)\) [ln(CFU/mL)], versus time (in hours) for experiments 1, 8, 21, 24 and 26 of dataset 1 (Table 6). Solid lines represent the fitted to data obtained with the model of Baranyi and Roberts [78]. These experiments were performed at different static pH conditions and without adding acetic acid to the medium.
**Figure 16:** Cell density data of *E. coli* K12 [ln(CFU)/mL] versus time [h] for different pH values (Δ: pH=4.80; □: pH 5.50; ○: pH 7.50; X: pH 8.50; +: pH 9.00). The solid lines represent the predictions of the Baranyi and Roberts model [78] for experiments of dataset 1 (Table 6) (— : pH 4.80; — — : pH 5.00; — : pH 7.50; — — : pH 8.50; — — : pH 9.00). Temperature was held at 37°C.

As it can be observed, all the yielded growth curves present clearly identifiable exponential and stationary phases. Due to the pre-cultures performed under the procedure described in section 3.2, cells had a very short adaptation to the experimental conditions. In fact, the existence of a lag phase can only be confirmed by fitting the model of Baranyi and Roberts [78] to the experimental data. As cells grew aerobically, the exponential phase could be identified during an experiment by a drop in the dissolved oxygen levels of the growth medium. This can be observed, e.g., in the dissolved oxygen profile of cells cultured at pH 7.50 (Figure 17). Near the end of the exponential phase, i.e., around 6 hours into the fermentation, the fact that cell numbers are close to its maximum value causes an even more pronounced decrease in the dissolved oxygen concentration. Figure 18 shows that, at this point, a relatively high amount of base was also added to the bioreactor to counteract a decrease in the pH. This reduction in the pH, however, was not higher than 0.06 pH units (Figure 19) and is generally attributed to a glucose uptake rate higher than its conversion to biomass and carbon dioxide, which results in an excess of acetic acid produced during the aerobic growth of bacteria [55].

When the exhaustion of an essential substrate from the medium occurs or a toxic metabolite produced by cells reaches inhibitory concentrations, growth ceases and the stationary phase can be identified by a plateau in the dissolved oxygen levels, as it can be seen from 6 hours in Figure 17. During the stationary phase of this cell culture, an increase of 0.10 pH units was also registered between the 8 and 9 hours of fermentation. This shift in pH was most likely caused by the depletion of glucose from the medium and by ceasing the production of acetic acid as a by-product of *E. coli* metabolism.
Instead, cells start consuming acetate, the salt form of acetic acid [55]. Large amounts of acid were added to set pH back to 7.50.

![Dissolved oxygen levels during the experiment performed at pH 7.50. These values report only to the beginning of the experiment.](image1)

**Figure 17:** Dissolved oxygen levels during the experiment performed at pH 7.50. These values report only to the beginning of the experiment.

![pH control through acid and base addition during the experiment carried out at pH 7.50. O: volume of acid (H₂SO₄) added to the bioreactor; Δ: volume of base (KOH) added to the bioreactor. These values report only to the beginning of the experiment and are cumulative. All previous acid and base volumes added to adjust the pH are not represented.](image2)

**Figure 18:** pH control through acid and base addition during the experiment carried out at pH 7.50. O: volume of acid (H₂SO₄) added to the bioreactor; Δ: volume of base (KOH) added to the bioreactor. These values report only to the beginning of the experiment and are cumulative. All previous acid and base volumes added to adjust the pH are not represented.
Figure 19: pH profile for cells cultured at pH 7.50, which was kept constant using a proportional-integral-derivative (PID) controller and compensating any deviation with the addition of acid (H₂SO₄) or base (KOH). These values report only to the beginning of the experiment.

An irregularity in bacterial growth was observed for cells growing at pH 9.00: 7 hours into the fermentation growth seems to have stopped, restarting only 2 hours later. To guarantee that it was not simply an error associated to viable cell counting, the dissolved oxygen profile for experiment 26 was examined (Figure 20). As it can be seen, dissolved oxygen levels remain constant during the same period as cell concentration, proving that growth was, indeed, destabilized. Previous studies suggest an early consume of acetate, which causes an increase of the external pH. This increase in pH leads to an additional stress, from which cells have to recover. Once the culture recovers and acetate levels drop, glucose consumption and acetate production restart, until glucose is completely depleted and the stationary phase is reached [100]. However, no H₂SO₄ was added (Figure 21) and pH remained practically constant (Figure 22) during the experiment. Instead, large amounts of base were added to the bioreactor at the same time the irregularity in growth was observed. This means that acetic acid was most likely produced, as a by-product of E. coli metabolism, and released to the medium over this period.

Everything considered, the observed irregularity in growth was most likely caused by the stressful pH conditions applied to the bioreactor. As mentioned in section 2.2.3, E. coli cells have the antiporter Ec-NhaA to counteract the increase in the cytoplasmic pH caused by the alkalinisation of the extracellular environment. However, the capacity of this antiporter to capture protons is thought to be at its limit at pH 9.00, which could explain the observed disturbances in growth [37].
**Figure 20**: Dissolved oxygen levels during the experiment performed at pH 9.00. These values report only to the beginning of the experiment.

**Figure 21**: pH control through acid and base addition during the experiment carried out at pH 9.00. ○: volume of acid (H₂SO₄) added to the bioreactor; △: volume of base (KOH) added to the bioreactor. These values report only to the beginning of the experiment and are cumulative. All previous acid and base volumes added to adjust pH are not considered.
Figure 22: pH profile for cells cultured at pH 9.00, which was kept constant using a PID controller and compensating any deviation with the addition of acid (H₂SO₄) or base (KOH). These values report only to the beginning of the experiment.

The parameters of the model of Baranyi and Roberts [78] were estimated following the routine described in section 3.7, in which the objective function of equation 45 is minimized. The 95% confidence intervals were calculated as a measure of the amount of trust that can be put in the correspondent estimates. To measure the quality-of-fit to data of the primary model used, the mean sum of squared errors (MSE, equation 50) was determined. All results are shown in Table 7.

Table 7: Parameter estimates, 95% confidence intervals and mean square error (MSE) from the fit of the model of Baranyi and Roberts [78] to the cell density data of experiments 1 (pH 4.80), 8 (pH 5.50), 21 (pH 7.50), 24 (pH 8.50) and 26 (pH 9.00) of dataset 1 (see Table 6).

<table>
<thead>
<tr>
<th>Nr. Experiment</th>
<th>n₀ (\text{ln}(\text{CFU}/\text{mL}))</th>
<th>λ [h]</th>
<th>μ max [h⁻¹]</th>
<th>n max (\text{ln}(\text{CFU}/\text{mL}))</th>
<th>MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.13 (±0.37)</td>
<td>1.70 (±1.10)</td>
<td>0.63 (±0.06)</td>
<td>18.58 (±0.24)</td>
<td>0.049</td>
</tr>
<tr>
<td>8</td>
<td>7.39 (±0.19)</td>
<td>0.68 (±0.06)</td>
<td>1.75 (±0.03)</td>
<td>21.00 (±0.10)</td>
<td>0.016</td>
</tr>
<tr>
<td>21</td>
<td>7.41 (±0.14)</td>
<td>0.63 (±0.10)</td>
<td>2.16 (±0.04)</td>
<td>21.46 (±0.08)</td>
<td>0.004</td>
</tr>
<tr>
<td>24</td>
<td>7.17 (±0.20)</td>
<td>0.68 (±0.14)</td>
<td>2.13 (±0.05)</td>
<td>21.21 (±0.10)</td>
<td>0.008</td>
</tr>
<tr>
<td>26</td>
<td>6.82 (±1.01)</td>
<td>0.11 (±1.64)</td>
<td>1.04 (±0.23)</td>
<td>15.81 (±0.60)</td>
<td>0.222</td>
</tr>
</tbody>
</table>

From Figure 16 and Table 7 it is possible to verify that the growth rate is probably at its maximum near pH 7.50, which was expected since E. coli is a neutrophil and the optimum pH values for this specific strain have been shown to be around 7.00-7.50 [94]. It can also be observed that the more acidic or more alkaline the environment is, the lower is the growth rate. As expected, the lowest growth rates were obtained for experiments 1 (pH 4.80) and 26 (pH 9.00), since these were the most
stressful pH conditions tested. Growth rate reduction also seems to be more pronounced near the growth boundaries, which have been reported to be around 4.50 (lower limit) and 9.00 (upper limit) [94, 101]. For the experiments performed at low pH conditions (1 and 8), it is possible, however, that the presence of relatively large amounts of undissociated acetic acid (Table 6) has an influence on the results. The undissociated form of organic acids is known to cause inhibition of metabolic functions by lowering the cytoplasmic pH [102, 103]. Therefore, caution should be taken when making considerations exclusively based on the pH effect for cells cultured under these conditions.

All parameter estimates have small confidence bounds and seem to be, therefore, reliable. The exceptions are \( n_0 \) for experiment 26, which presents a considerably large error in comparison with the remaining data, and the \( \lambda \) particularly at pH 4.80 and 9.00. In experiment 26, the error affecting the lag time is unacceptably high as it even surpasses the parameter itself. Even so, these standard deviations are not of major concern, as the focus is on the exponential phase, described by \( \mu_{\text{max}} \), which presents acceptable confidence intervals. The maximum population \( n_{\text{max}} \) which the medium can support appears to be also higher near neutral, optimal conditions and decreasing considerably for high and low pH values. Indeed, the effect of pH on the stationary phase of growth has long been reported [104].

In general, the low MSE values reveal the good fitting capacity of the model of Baranyi and Roberts [78] to the experimental data. As expected, the lowest MSE value was obtained for pH 7.50 (experiment 21). Optimal acetic acid concentration conditions correspond to its absence (i.e., 0.00 ppm). Therefore, in this experiment, both pH and undissociated acid conditions can be considered optimal and disturbances in growth are expected to occur only at more stressful conditions, as observed at pH 9.00.

The cell density data and primary model predictions for the remaining experiments used to test the effect of pH on the maximum specific growth rate can be found in Appendix.

### 5.2.2. Undissociated acetic acid effect on microbial growth

Figure 23 shows the natural logarithm of cell density measurements for *E. coli* K12 (triangles), \( n(t) \) [ln(CFU/mL)], versus time (in hours) for experiments 17 to 20 (Table 6). Solid lines represent the fit to data obtained with the model of Baranyi and Roberts [78]. In all these experiments, pH was kept at 6.50. This value is in the proximity of the optimum for growth, where pH and weak acids are known not to interact [105]. Acetic acid was added to the medium in different amounts, according to the experimental design in Figure 14.
Figure 23: Cell density data of *E. coli* K12 [ln(CFU/mL)] versus time [h] for different [HAc] values (Δ: [HAc]=2.81 ppm; □: [HAc]=26.73 ppm; ○: [HAc]=51.94 ppm; X: [HAc]=82.11 ppm). The solid lines represent the predictions of the Baranyi and Roberts model [78] (Δ: [HAc]=2.81 ppm; □: [HAc]=26.73 ppm; ○: [HAc]=51.94 ppm; X: [HAc]=82.11 ppm). pH was kept at 6.50 and temperature at 37°C.

All the yielded growth curves are as previously described. Baranyi and Roberts [78] model parameters were estimated by minimizing the difference between model predictions and the real microbial response (equation 45). The correspondent 95% confidence intervals were determined from the parameter variances (equation 46). The quality-of-fit of the primary model used was assessed by the mean sum of squared errors (MSE).

Table 8: Parameter estimates, 95% confidence intervals and mean square error (MSE) from the fit of the model of Baranyi and Roberts [78] to the cell density data of the experiments performed at pH 6.50 and different undissociated acetic acid concentrations: 17 ([HAc]=2.81 ppm), 18 ([HAc]=26.73 ppm), 19 ([HAc]=51.94 ppm) and 20 ([HAc]=82.11 ppm) (see Table 6).

<table>
<thead>
<tr>
<th>Nr. Experiment</th>
<th>$n_0$ [ln(CFU/mL)]</th>
<th>$\lambda$ [h]</th>
<th>$\mu_{max}$ [h$^{-1}$]</th>
<th>$n_{max}$ [ln(CFU/mL)]</th>
<th>MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>7.10 (±0.28)</td>
<td>0.66 (±0.18)</td>
<td>2.12 (±0.06)</td>
<td>21.45 (±0.14)</td>
<td>0.018</td>
</tr>
<tr>
<td>18</td>
<td>7.03 (±0.35)</td>
<td>1.13 (±0.28)</td>
<td>1.78 (±0.06)</td>
<td>21.54 (±0.20)</td>
<td>0.035</td>
</tr>
<tr>
<td>19</td>
<td>7.28 (±0.47)</td>
<td>1.53 (±0.53)</td>
<td>1.39 (±0.08)</td>
<td>21.48 (±0.31)</td>
<td>0.070</td>
</tr>
<tr>
<td>20</td>
<td>7.35 (±0.20)</td>
<td>2.02 (±0.40)</td>
<td>0.89 (±0.04)</td>
<td>21.44 (±0.14)</td>
<td>0.016</td>
</tr>
</tbody>
</table>

As a general remark, the Baranyi and Roberts model [78] was found to accurately fit the experimental data, resulting in low MSE values and accurate parameter estimations. Experiment 19 ([HAc]=51.94 ppm) has the largest confidence bounds and MSE due to the poorer quality of the data. As discussed in section 2.2.4, the inhibitory effect of organic acids is mainly caused by their lipophilic undissociated
form. In the cytoplasm, the dissociation of organic acid releases protons that decrease the intracellular pH (pH$_i$) interfering with the metabolic function. The parameter estimates from Table 8 suggest that the higher the concentration of undissociated acetic acid is, the more growth is inhibited. Unlike pH, growth under optimum conditions only occurs in the absence of undissociated acid (i.e., for a correspondent concentration of 0.00 ppm).

In this case, the maximum cell density $n_{max}$ appears to be independent on the amount of undissociated acid. This means that organic acids can delay microbial growth but not significantly influence the maximum population reached when the external pH is held constant. As expected, cells exposed to higher concentrations of undissociated acid presented longer lag times $\lambda$, since conditions are further from optimum.

Growth does not occur for concentrations higher than the minimum inhibitory concentration, which depends on the bacterial strain and on the acid used [96]. However, neither the cell density measurements, nor the estimated $\mu_{max}$ give any indication on its value, as no early induction of the stationary phase or significant decrease of the growth rate were observed.

The effect of acetic acid on the growth curves was also studied at more acidic pH environments, where the fraction of the undissociated form is known to be higher: pH 6.00, 5.50 and 5.00 (Figure 24). The curve described by the cell density measurements at pH 5.50 and 24.25 ppm of undissociated acid (experiment 8) was once again plotted for a better comparison between growth curves at different [HAc] values. At pH 5.00 and [HAc] = 69.19 ppm (Figure 24, C), the stationary phase was not observed as the experiment had to be stopped sooner than expected.
Figure 24: Cell density data of *E. coli* K12 (ln(CFU/mL)) versus time [h] for different [HAc] values and pH 6.00 (A), 5.50 (B) and 5.00 (C) (Δ: [HAc]=8.55 (A), 24.25 (B), 49.13 (C) ppm; □: [HAc]=36.92 (A), 43.59 (B), 57.30 (C) ppm; ◆: [HAc]=60.29 (A), 62.20 (B), 69.19 (C) ppm; X: [HAc]=93.54 (A), 100.13 (B), 80.16 (C) ppm; ⊕: [HAc]=101.83 (A), 89.34 (C) ppm; ♦: 90.52 (C) ppm). The solid lines represent the predictions of the Baranyi and Roberts model [78] predictions (→: [HAc]= 8.55 (A), 24.25 (B), 49.13 (C) ppm; ←: [HAc]=36.92 (A), 43.59 (B), 57.30 (C) ppm; ←: [HAc]=60.29 (A), 62.20 (B), 69.19 (C) ppm; ←: [HAc]=93.54 (A), 100.13 (B), 80.16 (C) ppm; ←: [HAc]=101.83 (A), 89.34 (C) ppm; ←: 90.52 (C) ppm). Temperature was kept at 37°C.

Just as in the previous cases, the parameters of the model of Baranyi and Roberts [78] were estimated by minimizing the sum of squared errors (equation 45) and are shown in Table 9 (pH 6.00), Table 10 (pH 5.50) and Table 11 (pH 5.00), along with the 95% confidence intervals and the MSE values for each experiment.
In general, the model of Baranyi and Roberts [78] presented a good quality-of-fit to the experimental data obtained from all the pH and undissociated acetic acid concentration conditions tested. However, for each pH value, the quality-of-fit decreased substantially for higher [HAc] values. Furthermore, the

### Table 9: Parameter estimates, 95% confidence intervals and mean square error (MSE) from the fit of the model of Baranyi and Roberts [78] to the cell density data of the experiments performed at pH 6.00 and different undissociated acetic acid concentrations: 12 ([HAc] = 8.55 ppm), 13 ([HAc] = 36.92 ppm), 14 ([HAc] = 60.29 ppm), 15 ([HAc] = 93.54 ppm) and 16 ([HAc] = 101.83 ppm) (see Table 6).

<table>
<thead>
<tr>
<th>Nr. Experiment</th>
<th>( n_0 \left[ \ln \left( \frac{CFU}{ml} \right) \right] )</th>
<th>( \lambda ) [h]</th>
<th>( \mu_{\text{max}} ) [h(^{-1})]</th>
<th>( n_{\text{max}} \left[ \ln \left( \frac{CFU}{ml} \right) \right] )</th>
<th>MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>7.19 (±0.25)</td>
<td>0.58 (±0.17)</td>
<td>1.94 (±0.05)</td>
<td>21.36 (±0.11)</td>
<td>0.015</td>
</tr>
<tr>
<td>13</td>
<td>7.08 (±0.25)</td>
<td>1.06 (±0.25)</td>
<td>1.51 (±0.04)</td>
<td>21.60 (±0.14)</td>
<td>0.017</td>
</tr>
<tr>
<td>14</td>
<td>7.13 (±0.29)</td>
<td>1.73 (±0.42)</td>
<td>1.13 (±0.05)</td>
<td>21.59 (±0.18)</td>
<td>0.029</td>
</tr>
<tr>
<td>15</td>
<td>7.08 (±0.24)</td>
<td>2.36 (±0.60)</td>
<td>0.75 (±0.05)</td>
<td>21.32 (±0.17)</td>
<td>0.023</td>
</tr>
<tr>
<td>16</td>
<td>7.18 (±0.45)</td>
<td>3.73 (±1.18)</td>
<td>0.65 (±0.05)</td>
<td>21.46 (±0.67)</td>
<td>0.124</td>
</tr>
</tbody>
</table>

### Table 10: Parameter estimates, 95% confidence intervals and mean square error (MSE) from the fit of the model of Baranyi and Roberts [78] to the cell density data of the experiments performed at pH 5.50 and different undissociated acetic acid concentrations: 8 ([HAc] = 24.25 ppm), 9 ([HAc] = 43.59 ppm), 10 ([HAc] = 62.20 ppm) and 11 ([HAc] = 100.13 ppm) (see Table 6).

<table>
<thead>
<tr>
<th>Nr. Experiment</th>
<th>( n_0 \left[ \ln \left( \frac{CFU}{ml} \right) \right] )</th>
<th>( \lambda ) [h]</th>
<th>( \mu_{\text{max}} ) [h(^{-1})]</th>
<th>( n_{\text{max}} \left[ \ln \left( \frac{CFU}{ml} \right) \right] )</th>
<th>MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>7.39 (±0.19)</td>
<td>0.68 (±0.06)</td>
<td>1.75 (±0.03)</td>
<td>21.00 (±0.10)</td>
<td>0.016</td>
</tr>
<tr>
<td>9</td>
<td>6.94 (±0.20)</td>
<td>1.43 (±0.26)</td>
<td>1.22 (±0.03)</td>
<td>20.88 (±0.12)</td>
<td>0.012</td>
</tr>
<tr>
<td>10</td>
<td>6.98 (±0.24)</td>
<td>1.59 (±0.40)</td>
<td>1.01 (±0.04)</td>
<td>20.86 (±0.15)</td>
<td>0.019</td>
</tr>
<tr>
<td>11</td>
<td>7.01 (±0.41)</td>
<td>2.16 (±0.89)</td>
<td>0.87 (±0.09)</td>
<td>21.09 (±0.19)</td>
<td>0.070</td>
</tr>
</tbody>
</table>

### Table 11: Parameter estimates, 95% confidence intervals and mean square error (MSE) from the fit of the model of Baranyi and Roberts [78] to the cell density data of the experiments performed at pH 5.00 and different undissociated acetic acid concentrations: 2 ([HAc] = 49.13 ppm), 3 ([HAc] = 57.30 ppm), 4 ([HAc] = 69.19 ppm), 5 ([HAc] = 80.16 ppm), 6 ([HAc] = 89.34 ppm) and 7 ([HAc] = 90.52 ppm) (see Table 6).

<table>
<thead>
<tr>
<th>Nr. Experiment</th>
<th>( n_0 \left[ \ln \left( \frac{CFU}{ml} \right) \right] )</th>
<th>( \lambda ) [h]</th>
<th>( \mu_{\text{max}} ) [h(^{-1})]</th>
<th>( n_{\text{max}} \left[ \ln \left( \frac{CFU}{ml} \right) \right] )</th>
<th>MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>7.11 (±0.32)</td>
<td>0.82 (±0.47)</td>
<td>1.04 (±0.04)</td>
<td>19.57 (±0.18)</td>
<td>0.029</td>
</tr>
<tr>
<td>3</td>
<td>6.93 (±0.26)</td>
<td>1.98 (±0.61)</td>
<td>0.79 (±0.05)</td>
<td>19.77 (±0.14)</td>
<td>0.025</td>
</tr>
<tr>
<td>4</td>
<td>6.90 (±0.34)</td>
<td>2.75 (±1.33)</td>
<td>0.65 (±0.16)</td>
<td>12.49 (±1.07)</td>
<td>0.041</td>
</tr>
<tr>
<td>5</td>
<td>6.94 (±0.22)</td>
<td>2.18 (±0.49)</td>
<td>0.84 (±0.05)</td>
<td>20.12 (±0.13)</td>
<td>0.018</td>
</tr>
<tr>
<td>6</td>
<td>7.14 (±0.29)</td>
<td>4.58 (±1.03)</td>
<td>0.51 (±0.03)</td>
<td>19.80 (±0.50)</td>
<td>0.063</td>
</tr>
<tr>
<td>7</td>
<td>6.97 (±0.37)</td>
<td>2.59 (±1.11)</td>
<td>0.56 (±0.04)</td>
<td>19.40 (±0.24)</td>
<td>0.066</td>
</tr>
</tbody>
</table>
\( \mu_{\text{max}} \) estimated for 80.16 ppm, at pH 5.00, is higher than that obtained in the presence of 69.19 or even 57.30 ppm of undissociated acid. This does not imply, however, that the \( \mu_{\text{max}} \) reduction is not proportional to \([HA]\). In fact, both this observation and the high MSE values are more likely to result from the poor quality of the data, as shown in Figure 24. Exposing cells to more stressful conditions (in this case, of acetic acid concentration) is known to increase the variability in the microbial response [57, 85, 106], a fact that might have contributed for the lower quality of the data obtained. However, it should not be excluded the hypothesis that the acetic acid measurements at low pH values present a low accuracy, affecting the growth rates of an additional error.

Although the maximum cell density reached is, in general, constant for each pH, it slightly decreases for more acidic environments. Experiment 4 was the exception, with an estimated \( n_{\text{max}} \) of around 12.50 ln(CFU/mL). However, this value results from having no data points describing the stationary phase, which is modelled by this parameter. The lower number of data points from the exponential phase in comparison with the other experiments and the uncertainty affecting \( n_{\text{max}} \) also led to a larger confidence interval on the \( \mu_{\text{max}} \) estimated for these conditions.

### 5.3. Secondary modelling

To describe *E. coli* K12 growth rate as a function of pH and undissociated acetic acid concentrations, the appropriate secondary model structure was selected. For this purpose, a sequential modelling approach was adopted. This approach states that combined effect models should be structured in a way that allows the identification of the separate effects and possible interactions [3]. For this reason, model building starts by selecting models from literature for pH and undissociated acetic acid that can be applied in a sequential modelling approach. These individual effect models are then compared in sections 5.3.1 and 5.3.2 to select the structures that best describe the microbial response to changes in pH and undissociated acetic acid concentration, respectively. Once the best-fitting models are selected, these are combined in a single structure to describe the overall effect of the two environmental factors considered on the growth rate of *E. coli* K12.

All models were compared not only by their applicability under a sequential modelling approach, but also considering more general criteria such as (i) simplicity, (ii) biological significance of the parameters, (iii) minimum number of parameters and (iv) quality-of-fit (measured by the MSE).
5.3.1. Selection of the best-fitting model to describe the effect of pH on the growth rate of *E. coli*

To model the separate effect of pH on the maximum specific growth rate, the 11 experiments of dataset 1 (Figure 15) were considered. These correspond to experiments performed without adding any acetic acid to the broth, even though it is known from the HPLC measurements that acetic acid is always present in the BHI. The model structures used to model this effect were the Cardinal pH Model (CPM) proposed by Rosso et al. [84] and its adaptations: srCPM and aCPM [3].

The CPM model (equation 26) [84] has four secondary model parameters with biological significance and no structural correlation. These are the theoretical minimum, optimum and maximum pH for growth ($pH_{\text{min}}$, $pH_{\text{opt}}$ and $pH_{\text{max}}$, respectively) and $\mu_{\text{opt}}$ [h⁻¹], the maximum specific growth rate at optimum pH conditions. In this model and both its adaptations, the growth rate is assumed to be zero for pH values lower than $pH_{\text{min}}$ and higher than $pH_{\text{max}}$. 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 (equation 27) [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. Figure 25 compares the maximum specific growth rates computed with the srCPM [3] for different $k$ values with the $\mu_{\text{max}}$ estimated using the primary model of Baranyi and Roberts [78]. In this plot, the intersection points of the model outputs with the abscissa axis for low and high pH values represent $pH_{\text{min}}$ and $pH_{\text{max}}$, respectively. The parameter estimates, 95% confidence intervals, mean sum of squared errors and the accuracy and bias factors obtained with the srCPM [3] model structure are shown in Table 12, for different $k$ values.
Figure 25: srCPM [3] output for different $k$ values: 2 (---), 3 (--), 4 (---), 6 (-- --). The growth rates computed using equation 30 are compared with the $\mu_{\text{max}}$ estimated using the model of Baranyi and Roberts model [78] with 95% confidence intervals ($\times$).

Table 12: Secondary model parameter estimates, 95% confidence intervals, mean square errors and the accuracy and bias factors of the srCPM [3] model (equation 27) tested for different $k$ values.

<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>$pH_{\text{min}}$ [-]</td>
<td>4.70 ($\pm$0.03)</td>
<td>4.77 ($\pm$0.01)</td>
<td>4.79 ($\pm$0.01)</td>
<td>4.796 ($\pm$0.003)</td>
<td>4.798 ($\pm$0.002)</td>
</tr>
<tr>
<td>$pH_{\text{opt}}$ [-]</td>
<td>7.08 ($\pm$0.09)</td>
<td>7.47 ($\pm$0.06)</td>
<td>7.72 ($\pm$0.09)</td>
<td>7.79 ($\pm$0.13)</td>
<td>7.90 ($\pm$0.18)</td>
</tr>
<tr>
<td>$pH_{\text{max}}$ [-]</td>
<td>9.48 ($\pm$0.13)</td>
<td>9.11 ($\pm$0.03)</td>
<td>9.04 ($\pm$0.02)</td>
<td>9.02 ($\pm$0.01)</td>
<td>9.01 ($\pm$0.01)</td>
</tr>
<tr>
<td>$\mu_{\text{opt}}$ [h$^{-1}$]</td>
<td>2.20 ($\pm$0.04)</td>
<td>2.21 ($\pm$0.02)</td>
<td>2.18 ($\pm$0.02)</td>
<td>2.13 ($\pm$0.03)</td>
<td>2.07 ($\pm$0.03)</td>
</tr>
<tr>
<td>MSE</td>
<td>0.183</td>
<td>0.092</td>
<td>0.131</td>
<td>0.199</td>
<td>0.286</td>
</tr>
<tr>
<td>$A_f$</td>
<td>1.046</td>
<td>1.028</td>
<td>1.025</td>
<td>1.032</td>
<td>1.040</td>
</tr>
<tr>
<td>$B_f$</td>
<td>0.997</td>
<td>0.998</td>
<td>0.999</td>
<td>0.999</td>
<td>0.998</td>
</tr>
</tbody>
</table>

As it can be observed in Figure 25, the estimated $\mu_{\text{max}}$ for the experiment performed at pH 9.00 is affected of a considerably high error, resultant from the disturbances that occurred during the exponential phase of the microbial population cultured under these conditions, as discussed in section 3.2.1. Even so, it was included in the dataset used for modelling the separate effect of pH to help estimating the cardinal parameter $pH_{\text{max}}$.

Increasing $k$ led to a consistent increase of $pH_{\text{opt}}$ and $pH_{\text{min}}$ and to lower $pH_{\text{max}}$ estimates region (Table 12). The result obtained was then a smaller growth region with a flatter region around the optimum pH for growth (Figure 25). This observation is in line with the structural modification introduced by Akkermans et al. [3] in the original model equation of the CPM [84] and with E. coli cells capacity to efficiently maintain pH homeostasis within the external pH range 6.50-8.50 [94]. Although the mechanisms behind pH homeostasis are not fully understood yet, this is suggested to result from a combination of passive and active strategies. A small decrease in the cytoplasmic pH can be
counteracted, e.g., by the presence of intracellular buffers such as the glutamate and aspartate side chains of proteins and the phosphate groups of nucleic acids [30], the low membrane permeability to protons itself and/or an active mechanism of proton extrusion through the membrane-bound transporter ATPase [28, 94].

Bacterial cells also possess pH homeostasis mechanisms that allow $\mu_{\text{max}}$ to remain almost constant despite small increases in the extracellular pH and, consequently, in the pH. These might include the production of acid metabolites through the activity of amino acid deaminases and sugar fermentation, an increased rate of proton transport to the cytoplasm by the ATP synthase or an increase in the proportion of unsaturated fatty acids at the membrane level to make it less rigid and more permeable to protons. In E. coli, this capacity has been related to a sodium/proton antiporter, the Ec-NhaA, that exchanges intracellular sodium cations for extracellular protons, decreasing the pH [37]. The existence of these mechanisms explains, then, why the maximum specific growth rate remains practically constant in the proximity of the optimum pH value, as observed in Figure 25.

Comparing the MSE values in Table 12, 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. This suggests that a flatter optimum does not necessarily correspond to a better description of microbial response to changes in the environmental pH. Indeed, both acid and alkaline pH homeostasis mechanisms are of limited capacity. At lower pH levels, literature suggests that the buffering capacity of the cells might be exceeded and the energy produced by the catabolic reactions within the cell is not sufficient to meet that demanded by the active pH homeostasis mechanisms [107]. An increasing $k$ value also led to a consistent decrease in $\mu_{\text{opt}}$ and to a more pronounced underestimation of the growth rates in the pH range 6.50-8.50, due to a more flattened optimum.

The most reliable estimation of the optimum pH for growth was obtained for $k$ equal to 3 and suggests that this is around 7.50, agreeing with the values reported in the literature for this E. coli strain [94]. For pH values higher than the optimum, the experimental $\mu_{\text{max}}$ also remains practically constant but only until a pH around 8.50 is reached. At this point, the major active mechanism of alkaline pH homeostasis, the sodium/proton antiporter Ec-NhaA, is thought to be very close to exceeding its capacity of capturing protons from the extracellular environment [37]. Even so, a difference to the suboptimal pH range is the fact that the plateau of maximum specific growth rates can be observed for pH values relatively close to the upper growth limits.

Furthermore, the most inhibitory form of the acetic acid (and organic acids in general), the undissociated form, was found to be present in high amounts for experiments performed in the suboptimal pH range (Figure 15). It is, indeed, very likely that high concentrations of undissociated acetic acid, $[HAc]$, led to an additional reduction of $\mu_{\text{max}}$ and, therefore, to a higher deviation from the flatter optimum described by higher values of $k$. The interference of $[HAc]$ on the growth rates
estimated for the experiments performed at lower pH conditions is discussed in more detail in section 4.3.3.

Analysing the accuracy factors displayed in Table 12, the lowest value calculated from equation 54 was obtained with \( k \) equal to 4. Therefore, it is this value of \( k \) that results in the lowest difference between the growth rates computed with the srCPM (equation 27) [3] and the growth rates estimated with the model of Baranyi and Roberts (equations 9 and 10) [78]. Nonetheless, accuracy factors are, in this case, calculated from the natural logarithm of \( \mu_{\text{max}} \) and the objective function of the one-step parameter estimation is the true microbial response. This is, in turn, involved in the computation of the sum of squared errors (SSE, equation 51) and, consequently, of the MSE (equation 50). The fact that the lowest MSE was obtained for \( k \) equal to 3 shows that, although \( \ln(\mu_{\text{max}}) \) is a valid approximation for most tested conditions, it might be that the best fit to the experimental growth rates does not always correspond to the best fit to the cell density data. The bias factors presented in Table 12 also suggest that applying the srCPM model structure [3] to the considered dataset results in practically insignificant underestimations of the growth rate.

The CPM [84] and srCPM [3] assume that the microbial population presents a structurally similar response to changes in the suboptimal and superoptimal pH ranges. However, as previously discussed, there are different factors influencing the acid and alkaline pH homeostasis mechanisms in *Escherichia coli*. In the aCPM (equation 28) [3], the factors for the suboptimal pH conditions are raised to a shape parameter \( \eta \) in both numerator and denominator. Just as with the shape parameter \( k \), the lack of biological significance of \( \eta \) makes its initial value estimation and convergence more difficult to achieve. For this reason, the \( \eta \) that provides the best description of microbial growth was also found by equalling 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]. All cardinal parameters were estimated simultaneously by achieving convergence with the *lsqnonlin* routine, as described in section 3.7. Figure 26 compares the maximum specific growth rates computed with the aCPM [3] for \( k \) equal to 3 and different \( \eta \) values with the \( \mu_{\text{max}} \) estimated using the model of Baranyi and Roberts [78]. The parameter estimates, 95% confidence intervals, mean sum of squared errors and the accuracy and bias factors obtained with the aCPM model structure [3] are shown in Table 13, for different \( \eta \) values.
From the parameter estimates in Table 13, it appears that an increase in the power for the suboptimal pH factors in the aCPM [3] model structure causes a decrease in all cardinal parameters to a certain extent. The exceptions are $pH_{max}$ and $\mu_{opt}$ when $\eta$ is raised from 4 to 5. However, this decrease in the cardinal parameter values is also followed by larger standard deviations, meaning that the amount of trust that can be put in these estimates reduces. For $\eta$ equal to 5, the maximum pH for growth is not even realistic, as the correspondent confidence interval largely exceeds the parameter value itself. This is caused by the lack of experimental data for pH values above 9.00. Moreover, as the experiment performed under those pH conditions is determinant for the estimation of $pH_{max}$, the large error associated to it results in a high uncertainty for $pH_{max}$ as well. Previous studies also suggest that *E. coli* K12 is not likely to grow for pH values higher than 9.00 [94]. As previously discussed, this is thought to be due to the very limited capacity of the main active pH homeostasis mechanism, the sodium/proton antiporter Ec-NhaA, to transport protons from the extracellular medium to the cytoplasm, so that the intracellular pH is reduced [37]. Considering the lower growth limits, as no...
experimental data for pH values lower than 4.80 was included in dataset 1 (Table 6), the further the estimated $pH_{min}$ is from that value, the higher the uncertainty on this cardinal parameter is. For this reason, the most and least accurate $pH_{min}$ estimates correspond to $\eta$ equal to 2 and 5, respectively (Table 13). The most accurate estimation of $pH_{opt}$ was also obtained for $\eta$ equal to 2 and is within the range of values suggested by the literature for E. coli K12 [94].

Further considerations are difficult to make as $\eta$ is a purely structural parameter and, therefore, cannot be interpreted from the biological point of view. 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. In this case, also the difference between the predicted growth rates and the experimental growth rates was minimized, as the lowest accuracy factor was obtained. The bias factors presented in Table 13 also suggest that applying the aCPM model structure [3] to the considered dataset results, in general, in practically insignificant underestimations (or overestimation, for $\eta$ equal to 5) of the growth rate.

The growth rates calculated using the CPM [84], the best-fitting srCPM [3] and the best-fitting aCPM [3] models are plotted in Figure 27. From visual comparison of the three cardinal parameter models, it can be observed that the CPM [3] has a mirror model structure around a distinct optimum, resultant from predicting an equal growth rate reduction for the suboptimal and superoptimal pH ranges. A consequence of this fact is that growth rates around the optimum pH region (6.50-7.50) were overestimated, whereas the growth rates at more acid or alkaline conditions were underestimated by the CPM [84]. This difference between the model predictions and the experimental growth rates is due to E. coli cells capacity to maintain pH homeostasis in the range 6.50-8.50 [94], as previously discussed. The existence of such homeostasis mechanisms is taken into account by the srCPM [3] with the inclusion of a shape parameter $k$. In general, including this structural parameter in the original CPM [84] equation and the consequent plateau observed in the proximity of the optimum pH resulted in better growth rate predictions for all the growth region. The extent of this plateau to more acid or alkaline conditions is thought to be essentially limited by the high energy requirements from the active pH homeostasis mechanisms and the inhibition of essential metabolic reactions [107]. Raising the factors for the suboptimal pH range to a structural parameter $\eta$ allowed a different description of the microbial response than that for the superoptimal range. As it can be observed in Figure 27, although the aCPM [3] also includes the structural adaptation of the srCPM [3], the additional parameter $\eta$ resulted in a less flat region around the optimum pH, which seems to provide a better adjustment to the experimental growth rates in the range of pH values 6.00-8.00.

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 14.
Figure 27: Comparison between different secondary models for the effect of pH on the maximum specific growth rate: 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 14: Secondary model parameter estimates, 95% confidence intervals, mean square errors and the accuracy and bias factors of the CPM [84] (equation 26), srCPM [3] (equation 27) and aCPM [3] (equation 28).

<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}}) [(\cdot)]</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}}) [(\cdot)]</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}}) [(\cdot)]</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}}) [(\text{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>
<tr>
<td>(A_f)</td>
<td>1.067</td>
<td>1.028</td>
<td>1.019</td>
</tr>
<tr>
<td>(B_f)</td>
<td>0.992</td>
<td>0.998</td>
<td>0.998</td>
</tr>
</tbody>
</table>

As expected, the model structure of the CPM [84] 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 [84]. 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%.

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. As it can be seen in Table 13, in dataset 1, used to model the separate effects of pH, were included experiments where no acetic
Acid had been added. However, high amounts of this acid were found in the BHI used for the growth medium (Table 6). As low pH conditions favour higher concentrations of the undissociated form of organic acids, this is very likely to have influenced the growth rates in the suboptimal range and, consequently, the value of $pH_{\text{min}}$. 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 (4.65) (data shown in Appendix). The data at pH 4.65 was not included in this dataset, since no HPLC measurement was performed for this experiment and simply assuming an average value for the acetic acid in the broth at such low pH could induce a large error. Furthermore, literature suggests that *E. coli* can grow at pH values as low as 4.40 [28]. 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 [84] model structures an extrapolation of the growth boundaries. It is also worth mentioning that all the estimated parameters representing the growth boundaries are theoretical. Therefore, to validate these estimates, experiments would have to be performed specifically for that purpose.

As expected, the optimal pH for growth was found to be in the range 7.00-7.50. The disturbances that occurred during the exponential phase and the low maximum population density presented by cells cultured at pH 9.00 (Figure 16) suggest that growth might be hardly sustainable at even more alkaline conditions. The estimated $pH_{\text{max}}$ values are, indeed, practically in line with these observations. CPM [84] 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. However, 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. [94] 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 [84] are correct. However, as it was previously mentioned, experiments in that pH range would have to be performed to validate its value. Parameters estimated using this model structure are also the most unrealistic, i.e., present the largest standard deviations, as no data was included for pH values below and above 4.80 and 9.00, respectively.

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].

### 5.3.2. Selection of the best-fitting model to describe the effect of undissociated acetic acid on the growth rate of *E. coli*

To assess the effect of undissociated acetic acid concentration on the maximum specific growth rate of *E. coli*, four different sets of experiments from Table 6 were initially considered: (i) experiments 2 to 7 (pH 5.00), (ii) experiments 8 to 11 (pH 5.50), (iii) experiments 12 to 16 (pH 6.00) and (iv)
experiments 17 to 20 (pH 6.50). All the mentioned sets have in common the fact that pH was kept constant in every experiment and that, in each set, the concentration of undissociated acetic acid varied between experiments.

As previously discussed in section 2.3.2.4, Presser et al. [9] has modelled the effect of lactic acid concentration, \([HAc] \text{ [ppm]}\), on the growth rate of \(E. \ coli \) M23. Although the dissociated form of the acid also presented an inhibitory potential, the undissociated form was reported to be much more effective under low pH conditions, typical of food products. In this model, it is assumed that \(\mu_{\text{max}} \text{ [h}^{-1}]\) decreases linearly with the increase in the concentration of the undissociated form of organic acids (equation 29) and that growth is limited by a minimum inhibitory concentration, \(MIC \text{ [ppm]}\). The \(MIC\) is constant for each bacterial strain. Le Marc et al. [2] proposed a different model structure, where the linear relationship of the growth rate was with the square root of the concentration of undissociated acetic acid instead (equation 30). This model was originally used to model the growth of \(Listeria \ monocytogenes\) [108].

In this research, no previous \(\mu_{\text{max}}-[HAc]\) relationship was initially assumed. Instead, a shape parameter \(\alpha [-]\) 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:

\[
\mu_{\text{max}}([HAc]) = \mu_{\text{opt}} \cdot (1 - \left(\frac{[HAc]}{MIC}\right)^\alpha)
\]

(56)

\[
\mu_{\text{max}}([HAc]) = \mu_{\text{opt}} \cdot (1 - \left(\frac{[HAc]}{MIC}\right)\alpha)
\]

(57)

In the proposed model structures, \(\mu_{\text{opt}} [\text{h}^{-1}]\) 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. Once \(\alpha\) is estimated, this can be fixed to a rounded value and used as such in a combined effect model. As the \(MIC\) is the smallest concentration of an acid capable of inhibiting growth, it was necessary to impose a restriction on the models equations: \(\mu_{\text{max}}([HAc]) = 0\) if \([HAc] \geq MIC\). The primary and secondary model parameters were estimated by the one-step method. Just as in section 4.3.1, \(\mu_{\text{max}}\) is not estimated as a primary model parameter, but calculated as a function of the undissociated acetic acid concentration using equations 56 and 57. Figure 28 compares the model predictions of equations 56 and 57 with the “measured” growth rates, i.e., estimated by the model of Baranyi and Roberts [78] (equations 9 and 10), at different pH values. The intersection point with the abscissas axis corresponds to the value of the \(MIC\) of the undissociated acid, whereas the intersection point with the ordinates axis gives the value for \(\mu_{\text{opt}}\).
Figure 28: Growth rate of *Escherichia coli* K12 [h⁻¹] versus undissociated acetic acid concentration [ppm] for experimental data collected at pH 6.50 (A), 6.00 (B), 5.50 (C), and 5.00 (D). Temperature was kept at 37°C. Solid lines represent the growth rates computed using the secondary models from equations 56 (—) and 57 (— —). Crosses correspond to the $\mu_{\text{max}}$ obtained by the fitting the model of Baranyi and Roberts [78] to the cell density measurements. Error bars were obtained from the 95% confidence intervals of the estimated maximum specific growth rates.

By comparing the growth rates calculated by both equations, the different position of the mathematical parameter $\alpha$ does not seem to determine significant differences between the outputs of both models, particularly at near optimal pH conditions, where data presents a better quality. A more relevant observation is that both model equations seem to result in a linear decrease of $\mu_{\text{max}}$ with the increase of $[\text{HAc}]$. At pH values near optimal (Figure 28, A and B), this linearity seems to result in a great fit to the experimental growth rates. The same observation does apply to more stressful pH values (Figure 28, C and D). However, the data collected under these conditions is of poorer quality and is expected to lead to an increased error.

Although estimated growth rates can be used to compare secondary model structures, in a one-step parameter estimation, model selection is based on the fit to the real microbial response. To each experiment there are three primary model parameters associated. Nonetheless, these are not shown in this study as the difference between the cell density measurements and the model predictions is
already reflected in the MSE value. The accuracy and bias factors were determined as described in section 3.7. It is assumed that the approximation used in the calculation of these factors is valid for the considered set of experimental conditions. All the results are shown in Table 15 and Table 16 for equations 56 and 57, respectively.

**Table 15:** Estimated minimum inhibitory concentration (MIC), α and μ\text{opt}, with μ\text{max}(HAc) = μ\text{opt} \cdot (1 - \frac{[HAc]}{MIC})^\alpha. Each parameter has a 95% confidence interval associated to. The mean sum of squared errors reflects the objective function of the one-step parameter estimation. Accuracy and bias factors computed from ln(μ\text{max}) are 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 [ppm]</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>α [-]</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>μ\text{opt} [h⁻¹]</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>
<tr>
<td>A_f</td>
<td>1.048</td>
<td>1.044</td>
<td>1.020</td>
<td>1.015</td>
</tr>
<tr>
<td>B_f</td>
<td>0.997</td>
<td>0.999</td>
<td>1.004</td>
<td>1.001</td>
</tr>
</tbody>
</table>

**Table 16:** Estimated minimum inhibitory concentration (MIC), α and μ\text{opt}, with μ\text{max}(HAc) = μ\text{opt} \cdot (1 - \frac{[HAc]}{MIC})^\alpha. Each parameter has a 95% confidence interval associated to. The mean sum of squared errors reflects the objective function of the one-step parameter estimation. Accuracy and bias factors computed from ln(μ\text{max}) are 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 [ppm]</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>α [-]</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>μ\text{opt} [h⁻¹]</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>
<tr>
<td>A_f</td>
<td>1.076</td>
<td>1.091</td>
<td>1.024</td>
<td>1.020</td>
</tr>
<tr>
<td>B_f</td>
<td>1.006</td>
<td>0.989</td>
<td>0.999</td>
<td>1.001</td>
</tr>
</tbody>
</table>

Both the computed mean sum of squared errors and the estimated parameters confirm that the position of the mathematical parameter α on the model structure does not have a significant influence on its output, as α was estimated to be very close to 1.00 for every pH value. An α 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. [9]. Different microorganisms have shown different levels of resistance to the stress caused by organic acids [2, 96]. The similarity of the obtained results with those of Presser et al. [9] can be justified by the fact that this model was also set up on *E. coli* growth data, whereas the model of Le Marc et al. [2] was
developed based on experimental observations of *Listeria monocytogenes* growth. However, further research is required before assuming that this model structure is valid for all *E. coli* strains. 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. It should also be mentioned that, in general, equation 56 led to larger standard deviations on $\mu_{opt}$ than equation 57, whereas the opposite was observed for the MIC and $\alpha$. Larger standard deviations also represent a lower sensitivity of the model to changes in a certain parameter. If this sensitivity is low, so is the confidence on the parameter. Therefore, the model structure of equation 56 showed up to be more sensitive to changes in the MIC and $\alpha$, whereas equation 57 is more sensitive to changes in $\mu_{opt}$.

An observation common for the results presented in both Table 15 and Table 16 is that the MSE consistently increases with lower pH values. Equations 56 and 57 assume that the undissociated acetic acid concentration is the only environmental factor influencing growth, as pH was kept constant in the entire set of experiments considered in this section. However, this consistent increase in the MSE value (and correspondent decrease in the quality-of-fit) 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 $[HAc]$ decreases. The higher accuracy factors were computed for pH 5.00 and 5.50 in both model structures, meaning that the poor quality of the data collected under these conditions also affected the accuracy with which microbial growth rates were estimated. This increase in the error with a decreasing pH could be, however, due either to (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 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. Whereas with equation 56, the MIC was computed to be around 113.55 ppm, equation 57 estimated its value to be 120.36 ppm. For this reason, the boundary defined by the smallest concentration of undissociated acetic acid that inhibits growth might not be independent from pH at more stressful levels of this environmental factor. It should be noted, however, that the estimated 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. The existence of interactions between pH and the concentration of undissociated acetic acid is discussed in more detail in section 4.3.3.

A trend could also be observed for $\mu_{opt}$, particularly in Table 16, 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 trend 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 $[HAc]$. Therefore, this corresponds to the parameter estimates at pH 6.50 of Table 16. Hereafter, the effect of undissociated acetic acid on the growth rate of *E. coli* K12 will also be referred to simply as the effect of the acetic acid concentration.

5.3.3. Modelling the combined effects of pH and undissociated acetic acid concentrations on the growth rate of *E. coli*

The simplest approach to model the combined effect of pH and undissociated acetic acid concentration on *E. coli* K12 growth rate is the gamma hypothesis (equation 32), which states that environmental factors act independently on microbial growth and that the overall model structure results from the multiplication of these factors. This implies a sequential modelling approach, where the separate effects of each hurdle can be clearly identified. However, 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 (Table 6). It is then likely that the individual effect of pH was not determined experimentally and, thus, is not truly described by the separate effect models and parameter estimates of Table 14.

In order to estimate the pure pH effect, a mathematical method involving the gamma value for different acetic acid concentrations, $\gamma([HAc])$, was used. First, $\gamma([HAc])$ was determined from the acetic acid model of equation 57, 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. Then, at different pH values, the $\mu_{max}$ that was calculated based on the parameter estimation with Baranyi and Roberts [78] (Table 7) was divided by the $\gamma([HAc])$ calculated from the acetic acid model. In the end, this yielded a set of data points where effect of acetic acid was basically removed, leaving just the pH effect. These data points can be visualized in the graph of Figure 29, but no model was fitted to this
data, as it is just to demonstrate the influence of the effect of acetic acid on the previous results and to get an idea of the pure pH effect on the growth rate. In this graph, the growth rate estimates for the pure pH effect are also compared with the “experimental” growth rates and the growth rates estimated with the aCPM model [3] of Table 14.

Figure 29: The set of growth rates yielded with the pure pH effect (Δ) is compared with growth rates computed by the aCPM model [3] of Table 14 (O) and the experimental growth rates (X), with the correspondent 95% confidence intervals (error bars).

The results obtained by removing the acetic acid effect show 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. A trend for the difference between the experimental growth rates and those for the pure pH effect can also be observed, as it consistently increases with lower pH values. These results also imply that the cardinal parameters estimated in section 4.3.1, particularly \( pH_{\text{min}} \), were highly influenced by the effect of acetic acid. In fact, if the aCPM [3] had been estimated on experiments with small amounts of acetic acid, higher growth rates would have been obtained in the suboptimal pH range. At high pH values, no difference between the experimental growth rates and those estimated for the pure pH effect was already expected to be observed as these conditions favour the presence of dissociated acetic acid, to which the microbial membrane is much less permeable.

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 14 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. In other words, a sequential modelling strategy, where the effects of pH and \([HAc]\) are completely
separated to build the secondary models of each effect and then test it on data that requires the combined effect, cannot really be applied. 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 (correspondent to dataset 3, in Figure 15). By applying the same validation strategy to the mathematical models with interactions, these can then be simply compared by the value of the MSE, which is a measure of the quality-of-fit.

As a benchmark, the gamma model was initially tested by combining the model structure of the aCPM [3] (equation 28) with the model for the separate effect of $[HAc]$ of equation 57, according to equation 32. Then, this was compared to a gamma model where the pH effect was described the srCPM [3] (equation 27) 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.

From the observations made in section 4.3.2, $\alpha$ was equalled to 1.00. To avoid problems during computations, the influence of $k$ and $\eta$ on the description of microbial growth was once again studied by attributing these shape parameters different fixed values. $\eta$ was initially set to 2, so that only the influence of $k$ on the model output could be studied. Once the value of $k$ that provides the best fit to the real microbial response was determined, this was fixed to that value so that only the influence of $\eta$ could be analysed. The results from Table 17 show that, in the non-interactive gamma model, the mean sum of squared errors is minimized when $k$ is equalled to 4, whereas, in section 4.3.1, this had been determined to be 3. 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 (Figure 29). For this reason, a higher value of $k$ seems logical when used in a combined effect model.

Table 17: Effect of the shape parameter $k$ on the MSE value obtained with the gamma model of equation 32, where $\gamma(E_i) = \frac{\mu_{\text{max}}(E_i)}{\mu_{\text{opt}}}$ (with $E_i$ being pH or $[HAc]$). $\mu_{\text{max}}(\text{pH})$ is either given by the aCPM [3] (equation 28) or by the srCPM [3] (equation 27). $\mu_{\text{max}}([HAc])$ corresponds to the undissociated acetic acid model of equation 57. $\eta$ 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$_{\text{aCPM}}$</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$_{\text{srCPM}}$</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 18: Effect of the shape parameter $\eta$ on the MSE value obtained with the gamma model of equation 32, where $\gamma(E_i) = \mu_{max}(E_i)/\mu_{opt}$ (with $E_i$ being pH or [HAc]). $\mu_{max}(pH)$ and $\mu_{max}([HAc])$ are given by the aCPM [3] (equation 32). $\mu_{max}([HAc])$ corresponds to the undissociated acetic acid model of equation 57. $k$ was fixed to 4, based on previous observations.

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

Based on the lower MSE values presented in Table 17, it is shown that the model for the combined effect is improved by using the srCPM [3] as the gamma factor for the pH effect. Indeed, increasing the value of $\eta$ in the global model structure obtained from the aCPM [3] only led to higher MSE values. This observation is in line with the assumption that the pure pH effect should include higher growth rates at low pH values and that, therefore, the pure pH model should have a more extended plateau in the suboptimal range, which can be expressed by a model such as the srCPM [3].

The previous approach assumes that pH and [HAc] act independently, so that they can be combined in a multiplicative way in secondary models. Ross et al. [10] developed a model for the effects of temperature, water activity, pH and lactic on the growth rate of E. coli, stating that the gamma hypothesis is valid for a wide range of conditions. More recent studies have also been unable to prove the existence of interactions specifically between pH and weak acids for other microorganisms that not E. coli [96, 109, 110]. However, for the gamma concept to be truly valid, it is necessary that the value of typical model parameters, when modelling the separate effects of one factor, are not influenced by more stressful levels of other environmental factors. For the specific case of the undissociated acetic concentration, this means that the MIC should not be dependent on pH. As discussed in section 4.3.2, the MIC was, indeed, estimated to be practically constant between pH 5.00 and 6.50. Nonetheless, at pH 5.00, closer to the lower growth boundary, the MIC obtained was around 25 to 30 ppm lower, depending on the model structure. This might support the hurdle theory [19] in the sense that cells exposed to pH conditions near the growth boundaries are more sensitive to the action of the undissociated acetic acid, resulting in an inhibitory effect greater than that expected from the multiplication of the individual gamma factors. However, as it was also mentioned in section 4.3.2, it should be noted that this lower MIC value at pH 5.00 is affected of a high uncertainty, possibly due to the low accuracy of the acetic acid measurements at low pH. If this is the case, then the measured growth rates are at different [HAc] values and, therefore, the MIC value obtained at pH 5.00 cannot be used to prove the existence of interactions.

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 of Akkermans et al. [3], based on dataset 3 of Figure 15. Both the models of Augustin and Carlier [1] (equations 33 to
35) and Akkermans et al. [3] (equations 42 and 43) include an additional parameter $\beta [\cdot]$, in comparison with the nonsynergistic gamma model, which can be related to the extent of interactions. In their study, Augustin and Carlier [1] gave $\beta$ a value of 3, based on a set of published growth/"no-growth" data. In this research, however, $\beta$ was estimated along with the other secondary model parameters for a better description of the combined effect of pH and undissociated acetic acid concentration. On the other hand, the model of Le Marc et al. [2] presents the exact same parameters as the non-interactive gamma model, which means that interactions are exclusively defined based on the separate effects and are assumed to occur only at very stringent conditions.

Figure 30 shows the three-dimensional representations of the experimental growth rates and of the growth rates computed by the four combined effect models [h$^{-1}$] versus pH [-] and undissociated acetic acid concentration [ppm]. Parameters were estimated directly on cell density measurements by the one-step method. The results of the parameter estimation along with the MSE, $A_f$ and $B_f$ values are summarized in Table 19.

![Figure 30](image-url)

**Figure 30**: Three-dimensional representation of the experimental growth rates and of the growth rates computed by the four combined effect models [h$^{-1}$] versus pH [-] and undissociated acetic acid concentration [ppm]: gamma model (A), model of Augustin and Carlier [1] (B), model of Le Marc et al. [2] (C) and gamma-interaction model [3] (D).
Table 19: Parameter estimates and their 95% confidence intervals, MSE, \( A_f \) and \( B_f \) values for the nonsynergistic 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>
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</thead>
<tbody>
<tr>
<td>( pH_{\text{min}} ) [-]</td>
<td>4.64 (± 0.05)</td>
<td>4.39 (± 0.14)</td>
<td>4.65 (± 0.05)</td>
<td>4.36 (± 0.37)</td>
</tr>
<tr>
<td>( pH_{\text{opt}} ) [-]</td>
<td>7.63 (± 0.13)</td>
<td>7.70 (± 0.13)</td>
<td>7.62 (± 0.13)</td>
<td>7.67 (± 0.13)</td>
</tr>
<tr>
<td>( pH_{\text{max}} ) [-]</td>
<td>9.04 (± 0.02)</td>
<td>9.034 (± 0.02)</td>
<td>9.04 (± 0.02)</td>
<td>9.03 (± 0.02)</td>
</tr>
<tr>
<td>( MIC ) [ppm]</td>
<td>145.53 (± 2.03)</td>
<td>149.84 (± 2.58)</td>
<td>145.52 (± 2.03)</td>
<td>152.08 (± 7.99)</td>
</tr>
<tr>
<td>( \mu_{\text{opt}} ) [h(^{-1})]</td>
<td>2.21 (± 0.04)</td>
<td>2.18 (± 0.03)</td>
<td>2.21 (± 0.03)</td>
<td>2.20 (± 0.13)</td>
</tr>
<tr>
<td>( \beta ) [-]</td>
<td>-</td>
<td>12.44 (± 3.99)</td>
<td>-</td>
<td>1.33 (± 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>
<tr>
<td>( A_f )</td>
<td>1.065</td>
<td>1.029</td>
<td>1.065</td>
<td>1.066</td>
</tr>
<tr>
<td>( B_f )</td>
<td>0.984</td>
<td>0.996</td>
<td>1.016</td>
<td>1.019</td>
</tr>
</tbody>
</table>

As it can be seen, the interaction factor \( \gamma_r(pH, [HAc]) \) 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. Equation 36 shows that when \( \gamma_r(pH, [HAc]) \) is equal to 1.0, the model structure proposed by Le Marc et al. [2] is reduced to the gamma equation, where the combined effect of pH and \([HAc]\) results strictly from the multiplication of the individual gamma factors, \( \gamma(pH) \) and \( \gamma([HAc]) \). For this interaction model and the lowest pH value tested (4.80), the lowest undissociated acetic acid concentration predicted to yield a \( \gamma_r(pH, [HAc]) \) value lower than 1.0 was 101.51 ppm, where no experimental data is available.

When the combined effect was described by the synergistic model of Augustin and Carlier [1], the MSE value presented a very small decrease of 3%. Generally, lower values of \( \beta \) result in smaller growth regions and, consequently, also in lower growth rates. In this sense, the value of this shape parameter is an indicative of the extent of the interactions. However, in this case, 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 does not clearly prove the existence of interactions between pH and \([HAc]\). Instead of including the information on (possible) interactions in the gamma equation by a gamma interaction factor, the model of Augustin and Carlier [1] includes this information on the growth limits, making \( pH_{\text{min}} \) and the \( MIC \) also dependent on \([HAc]\) and pH, respectively (equations 33 and 34). For this reason, the \( pH_{\text{min}} \) 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. It can, indeed, be seen as an approximation, but it can only be validated by performing experiments specifically for that purpose.

With the gamma-interaction model of Akkermans et al. [3], the MSE was reduced in 6%, comparing to the nonsynergistic gamma model. This model was already expected to perform, at least, as well as the gamma model, because if the parameter estimation procedure sets $\beta$ to 0.00, the interaction factor becomes equal to 1.0, reducing the model structure to that of the gamma model. The only (very small) difference in the MSE is, then, caused by the existence of an additional parameter in the model structure. This difference is not very significant since the amount of measurements and parameters are both high. Everything considered, the influence of $\beta$ on the model output is only expected for more stringent conditions, where both gamma factors for the separate effects of pH and $[HAc]$ are lower than one. Generally, from the way this model was built, the value of $\beta$ can also be used to interpret the extent of interactions, even though it is devoid of any biological meaning, with values closer to 0.00 representing the non-occurrence of interactions and large positive values representing the existence of strong interactions between environmental conditions. However, the fact that $\beta$ was estimated with large confidence intervals, that almost exceed the parameter value itself, suggests that the model is not very sensitive to $\beta$ in this case.

**Figure 31** shows that for the effect of pH, $\gamma_i (pH, [HAc])$ 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 very low values for high organic acid concentrations, but there is no sufficient data to support statements about interactions at this level.

![Figure 31: Interaction factor $\gamma_i$ [-] of the gamma-interaction model of Akkermans et al. [3] as a function of pH [-] and $[HAc]$ [ppm].](image-url)
Figure 32 compares the growth rates calculated with the gamma-interaction [3] and nonsynergistic gamma models with the ones estimated with the model of Baranyi and Roberts [78] at more stressful pH conditions (pH 5.00), where the differences between the gamma-interaction [3] model and the nonsynergistic gamma model were more pronounced. As it is shown for the considered set of data points, the prediction of lower growth rates at lower pH values resulted in a slightly better approximation to the experimental growth rates. This difference is, however, very small (less than 0.04 h⁻¹), and only improves the approximation to the “measured” growth rates in experiments with a large error on the growth rate. Therefore, 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. For this reason, future research on the occurrence of synergistic effects between pH and organic acids, such as the acetic acid, is required.

![Figure 32: Comparison between experimental growth rates (X), with the correspondent 95% confidence intervals, the growth rates calculated with the gamma-interaction model (Δ) and nonsynergistic gamma model (O) at pH 5.00.](image-url)
6. Conclusions and future perspectives

At more stressful conditions, certain environmental factors may present synergistic interactions and produce an overall inhibitory effect higher than that expected from the separate constraints [19]. Two factors extensively used for food preservation and closely related by definition are the environmental pH and the organic acid concentration. 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 microbial growth dynamics, particularly on the maximum specific growth rate $\mu_{\text{max}}$, in an accurate way. An emphasis was given on the validity of the gamma concept, which assumes that environmental factors act independently of each other. To this end, a set of 26 bioreactor experiments was performed with *E. coli* K12 under static conditions.

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. The aCPM [3] outperformed both the CPM [84] and srCPM [3] in modelling the separate effect of pH, but it was the gamma model where the gamma factor for pH was given by the srCPM [3] (which presents a more extended plateau in the suboptimal pH range) that presented the best quality-of-fit. 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.

In turn, it might be more difficult to experimentally separate the effects of pH and acetic acid. One option could be to prepare the growth medium from its separate components but it is quite uncertain whether acetic acid could not be produced from a reaction in the medium involving any of these components. Further research on the growth limits is required as well. However, the increased variability in the behavior of the microbial population at more stressful conditions makes it difficult to obtain good quality data near the growth/no-growth boundaries. The $pH_{\text{max}}$ estimated with the aCPM [3], with an approximate value of 9.00, was quite accurate and in line with the irregularities observed at this pH and with the values reported in the literature for *E. coli* K12 [3, 94], but experiments should be performed at slightly higher pH conditions to validate this estimate. Nonetheless, it is in the suboptimal region that most food products have their pH. Therefore, obtaining accurate $pH_{\text{min}}$ estimates is of the utmost importance. However, near the lower growth limits, obtaining reliable data and accurate parameter estimates can prove to be particularly challenging if the effects of pH and acetic acid are not separated, since the increased variability in the microbial response caused by low
pH itself can be further intensified by the relatively high ratio of undissociated organic acid in this region.

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. This consistency in the results was clearly affected for sets of experiments performed at a lower pH. Modelling $\mu_{max}$ as a function of $[HAc]$, supporting the statement of Presser et al. [9], which was made based on growth data for a different $E. coli$ strain and lactic acid. Other authors obtained different descriptions of the effect of the undissociated form of different organic acids on the microbial growth rate of microorganisms other than $E. coli$ [2, 96]. Therefore, it can be useful to test if the relationship between $\mu_{max}$ and $[HA]$ proposed by Presser et al. [9] and observed in this case study is also applicable for $E. coli$ strains in general and in response to other organic acids.

The inhibitory potential of organic acids is characterized by their minimum inhibitory concentration, $MIC$. 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. Two different explanations were proposed to justify the poorer quality of the data (and of the fit), with the most likely being a low accuracy of the HPLC measurements at lower pH conditions, meaning that the growth rates could have been estimated at $[HAc]$ values different than those that were considered and that, therefore, the $MIC$ value at pH 5.00 is not correct. To uncover this issue in future research, one option is to perform more replicates of the HPLC measurements and/or to repeat the experiments with larger standard deviations.

The validity of the gamma model was tested by comparison with 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]. Given the current dataset, the gamma hypothesis could not be refuted. 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]. The improvement in the quality-of-fit is, then, highly questionable and does not really support the occurrence of interactions. Great caution should be taken when assuming the existence of synergies where these might be, indeed, absent, since an incorrect estimation of the growth boundaries can put the safety of a food product at risk.

**Future perspectives:** Everything considered, future research on interactions between pH and organic acids, such as acetic acid, is required and should focus on conditions where the gamma factors of both pH and acetic acid concentration are clearly below one (i.e., at lower pH values and higher undissociated acid concentrations). Otherwise, the effects of the gamma-interaction model will still be difficult to identify. 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 in experiments performed at more extreme pH and organic acid concentration 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 ultimate goal 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.
References


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


Appendix

A. Fitted growth curves and parameter estimation with the Baranyi and Roberts model

A.1. Experiment 21 (pH 7.00; \([HAc]\) = 0.98 ppm)

![Graph showing growth of E. coli K12 at pH 7 and \([HAc]\) = 0.98 ppm.](image)

**Figure A.1**: Growth of *E. coli* K12 at pH 7 and \([HAc]\)=0.98 ppm. Cell density measurements \([\ln(CFU/mL)]\) (Δ) are represented as a function of time [h]. The solid blue line gives the fit obtained with the model of Baranyi and Roberts [78].

**Table A.1**: Parameter estimates, 95% confidence intervals and mean square error (MSE) from the fit with Baranyi and Roberts model [78] for experiment 21 (pH 7 and \([HAc]\)=0.98 ppm).

<table>
<thead>
<tr>
<th>Nr. Experiment</th>
<th>(n_0) [(\ln(CFU/mL))]</th>
<th>(q_0) [-]</th>
<th>(\mu_{max}) [h(^{-1})]</th>
<th>(n_{max}) [(\ln(CFU/mL))]</th>
<th>MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>6.978 (±0.176)</td>
<td>0.540 (±0.114)</td>
<td>2.196 (±0.041)</td>
<td>21.446 (±0.253)</td>
<td>0.007</td>
</tr>
</tbody>
</table>
A.2. Experiment 23 (pH 8.00; \([HAc] = 0.10 \text{ ppm}\))

**Figure A.2**: Growth of *E. coli* K12 at pH 8 and \([HAc]=0.10 \text{ ppm}\). Cell density measurements \([\ln(CFU/mL)] \ (\Delta)\) are represented as a function of time [h]. The solid blue line gives the fit obtained with the model of Baranyi and Roberts [78].

**Table A.2**: Parameter estimates, 95% confidence intervals and mean square error (MSE) from the fit with Baranyi and Roberts model [78] for experiment 23 (pH 8 and \([HAc]=0.10 \text{ ppm}\)).

<table>
<thead>
<tr>
<th>Nr. Experiment</th>
<th>(n_0 \ [\ln(CFU/mL)])</th>
<th>(q_0 \ [-])</th>
<th>(\mu_{\text{max}} \ [h^{-1}])</th>
<th>(n_{\text{max}} \ [\ln(CFU/mL)])</th>
<th>MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>7.251 (±0.226)</td>
<td>0.577 (±0.157)</td>
<td>2.234 (±0.063)</td>
<td>21.330 (±0.102)</td>
<td>0.011</td>
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</tbody>
</table>
A.3. Experiment 25 (pH 8.75; $[HAc] = 0.02$ ppm)

![Graph showing growth of E. coli K12 at pH 8.75 and $[HAc]=0.02$ ppm. Cell density measurements $[ln(CFU/mL)]$ are represented as a function of time $[h]$. The solid blue line gives the fit obtained with the model of Baranyi and Roberts [78].]

**Figure A.3:** Growth of *E. coli* K12 at pH 8.75 and $[HAc]=0.02$ ppm. Cell density measurements $[ln(CFU/mL)]$ (Δ) are represented as a function of time [h]. The solid blue line gives the fit obtained with the model of Baranyi and Roberts [78].

**Table A.3:** Parameter estimates, 95% confidence intervals and mean square error (MSE) from the fit with Baranyi and Roberts model [78] for experiment 25 (pH 8 and $[HAc]=0.10$ ppm).

<table>
<thead>
<tr>
<th>Nr. Experiment</th>
<th>$n_0$ $[ln(CFU/mL)]$</th>
<th>$q_0$ [-]</th>
<th>$\mu_{max}$ $[h^{-1}]$</th>
<th>$n_{max}$ $[ln(CFU/mL)]$</th>
<th>MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>7.199 $(\pm 0.252)$</td>
<td>0.967 $(\pm 0.195)$</td>
<td>1.898 $(\pm 0.054)$</td>
<td>20.655 $(\pm 0.101)$</td>
<td>0.017</td>
</tr>
</tbody>
</table>
A.4. Additional experiment (pH 4.65)

Figure A.4: Growth of *E. coli* K12 in an additional experiment at pH 4.65. Cell density measurements [ln(CFU/mL)] (Δ) are represented as a function of time [h]. The solid blue line gives the fit obtained with the model of Baranyi and Roberts [78].

Table A.4: Parameter estimates, 95% confidence intervals and mean square error (MSE) from the fit with Baranyi and Roberts model [78] for an additional experiment performed at pH 4.65.

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<thead>
<tr>
<th>pH</th>
<th>$n_0$ [ln(CFU/mL)]</th>
<th>$q_0$ [-]</th>
<th>$\mu_{max}$ [h$^{-1}$]</th>
<th>$n_{max}$ [ln(CFU/mL)]</th>
<th>MSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.65</td>
<td>7.063 (± 0.279)</td>
<td>0.784 (± 0.794)</td>
<td>0.620 (± 0.041)</td>
<td>18.071 (± 0.149)</td>
<td>0.024</td>
</tr>
</tbody>
</table>

B. Sensitivity equations

Natural logarithmic transformation of the Baranyi and Roberts equations [78]:

\[
\frac{dN(t)}{dt} = \frac{Q(t)}{1 + Q(t)} \cdot \mu_{max} \cdot \left(1 - \frac{N(t)}{N_{max}}\right) \cdot N(t) \tag{B.1}
\]

\[
\frac{dQ(t)}{dt} = \mu_{max} \cdot Q(t) \tag{B.2}
\]

By definition, $N(t) = \exp(n(t))$, $Q(t) = \exp(q(t))$ and $N_{max} = \exp(n_{max})$. Therefore, the natural logarithmic transformation of the Baranyi and Roberts model equations [78] is as follows:
\[
\frac{dn(t)}{dt} = \frac{1}{1 + \exp(-q(t))} \cdot \mu_{\text{max}} \cdot (1 - \exp(n(t) - n_{\text{max}})) \tag{B.3}
\]

\[
\frac{dq(t)}{dt} = \mu_{\text{max}} \tag{B.4}
\]

**Fisher information matrix** [97] and sensitivity equations: The Fisher information matrix \(F\) contains the variance \(s_n^2\) and the sensitivity of the model output, \(n(t)\), to a set of parameters \(p\). For two generic parameters, \(p_i\) and \(p_j\):

\[
F = \frac{1}{s_n^2} \int_0^t \begin{bmatrix}
\frac{(dn(t))^2}{dp_i} & \frac{(dn(t))}{dp_i} \cdot \frac{(dn(t))}{dp_j} \\
\frac{(dn(t))}{dp_i} \cdot \frac{(dn(t))}{dp_j} & \frac{(dn(t))^2}{dp_j^2}
\end{bmatrix} dt
\]

\[
\frac{dn(t)}{dp_i} = \int_0^t \frac{d}{dt} \left( \frac{dn(t)}{dp_i} \right) dt \tag{B.6}
\]

\[
\frac{dq(t)}{dp_i} = \int_0^t \frac{d}{dt} \left( \frac{dq(t)}{dp_i} \right) dt \tag{B.7}
\]

\[
\frac{d}{dt} \left( \frac{dq(t)}{dp_i} \right) = \frac{d}{dp_i} \left( \frac{dq(t)}{dt} \right) = \frac{d\mu_{\text{max}}}{dp_i} \tag{B.8}
\]

\[
\frac{d}{dt} \left( \frac{dn(t)}{dp_i} \right) = \frac{d}{dp_i} \left( \frac{dn(t)}{dt} \right) = \frac{d}{dp_i} \left( \frac{1}{1 + \exp(-q(t))} \cdot \mu_{\text{max}} \cdot (1 - \exp(n(t) - n_{\text{max}})) \right) \tag{B.9}
\]

For the natural logarithm of initial cell concentration \((n_0)\), with \(\frac{d\mu_{\text{max}}}{dn_0} = 0\) and \(\frac{dq(t)}{dn_0} = 0\):

\[
\frac{d}{dt} \left( \frac{dn(t)}{dn_0} \right) = \frac{d}{dn_0} \left( \frac{1}{1 + \exp(-q(t))} \cdot \mu_{\text{max}} \cdot (1 - \exp(n(t) - n_{\text{max}})) \right) \tag{B.10}
\]

\[
= -\mu_{\text{max}} \cdot \frac{1}{1 + \exp(-q(t))} \cdot \exp(n(t) - n_{\text{max}}) \cdot \frac{dn(t)}{dn_0}
\]

For the natural logarithm of initial physiological state of cells \((q_0)\), with \(\frac{d\mu_{\text{max}}}{dq_0} = 0\):

\[
\frac{d}{dt} \left( \frac{dq(t)}{dq_0} \right) = \frac{d\mu_{\text{max}}}{dq_0} = 0 \tag{B.11}
\]
\[
\frac{d}{dt} \left( \frac{dn(t)}{dq_0} \right) = \frac{d}{dq_0} \left( \frac{1}{1 + \exp(-q(t))} \cdot \mu_{\text{max}} \cdot (1 - \exp(n(t) - n_{\text{max}})) \right) \\
= \mu_{\text{max}} \left[ -\left( \frac{1}{1 + \exp(-q(t))} \right) \cdot \exp(n(t) - n_{\text{max}}) \cdot \frac{dn(t)}{dq_0} \right. \\
\left. + \left( \frac{1}{(1 + \exp(-q(t))^2} \right) \cdot (1 - \exp(n(t) - n_{\text{max}})) \cdot \frac{dq(t)}{dq_0} \right]
\]  
(B.12)

For the natural logarithm of maximum cell density \( n_{\text{max}} \), with \( d\mu_{\text{max}}/dn_{\text{max}} = 0 \):

\[
\frac{d}{dt} \left( \frac{dn(t)}{dn_{\text{max}}} \right) = \frac{d}{dn_{\text{max}}} \left( \frac{1}{1 + \exp(-q(t))} \cdot \mu_{\text{max}} \cdot (1 - \exp(n(t) - n_{\text{max}})) \right) = \\
\mu_{\text{max}} \cdot \frac{1}{1 + \exp(-q(t))} \cdot \exp(n(t) - n_{\text{max}}) \cdot (1 - \frac{dn(t)}{dn_{\text{max}}})
\]  
(B.13)

For any secondary model parameter \( p_i \), where \( f_i \) is the Baranyi and Roberts equation [78] and \( \frac{d}{dp_i} \left( \frac{dq(t)}{dt} \right) = \frac{d\mu_{\text{max}}}{dp_i} \):

\[
\frac{d}{dt} \left( \frac{dn(t)}{dp_i} \right) = \frac{df_1}{dn} \cdot \frac{dn}{dp_i} + \frac{df_1}{dq} \cdot \frac{dq}{dp_i} + \frac{df_1}{dp} \cdot \frac{dp}{dp_i} = g_1 + g_2 + g_3
\]  
(B.14)

\[
g_1 = -\left( \frac{1}{1 + \exp(-q(t))} \right) \cdot \mu_{\text{max}} \cdot \exp(n(t) - n_{\text{max}}) \cdot \frac{dn(t)}{dp_i}
\]  
(B.15)

\[
g_2 = \left( \frac{\exp(-q(t))}{(1 + \exp(-q(t))^2} \right) \cdot \mu_{\text{max}} \cdot (1 - \exp(n(t) - n_{\text{max}})) \cdot \frac{dq(t)}{dp_i}
\]  
(B.16)

\[
g_3 = \left( \frac{1}{1 + \exp(-q(t))} \right) \cdot (1 - \exp(n(t) - n_{\text{max}})) \cdot \frac{d\mu_{\text{max}}}{dp_i}
\]  
(B.17)

The derivative of the secondary model equations, where \( \mu_{\text{max}} \) is a function of the environmental conditions, to the secondary model parameters \( p_i \) gives the expression for \( d\mu_{\text{max}}/dp_i \). The sensitivity of the model output to (small) changes in the value of each parameter \( p_i \) is obtained by integrating the previous equations. As an initial condition for the integration step, all the partial derivatives were equalled to 0 at time \( t = 0 \). The exceptions were \( dn/dn_0 \) and \( dq/dq_0 \), which are both equal to 1 at \( t = 0 \), from the definition of initial cell concentration and initial physiological state.
Considering a single experiment and using the Cardinal pH Model of Rosso et al. [84] as an example, the Jacobian matrix containing the partial derivatives of the model output, \( n(t) \), at different sampling times, is given by:

\[
J_1 = 
\begin{bmatrix}
\frac{dn(t = t_1)}{dpH_{\text{min}}} & \frac{dn(t = t_1)}{dpH_{\text{opt}}} & \frac{dn(t = t_1)}{dpH_{\text{max}}} & \frac{dn(t = t_2)}{du_{\text{opt}}} & \frac{dn(t = t_1)}{d\mu_{\text{opt}}} & \frac{dn(t = t_2)}{dn_0} & \frac{dn(t = t_2)}{d\mu_0} \\
\frac{dn(t = t_2)}{dpH_{\text{min}}} & \frac{dn(t = t_2)}{dpH_{\text{opt}}} & \frac{dn(t = t_2)}{dpH_{\text{max}}} & \frac{dn(t = t_3)}{du_{\text{opt}}} & \frac{dn(t = t_2)}{d\mu_{\text{opt}}} & \frac{dn(t = t_3)}{dn_0} & \frac{dn(t = t_3)}{d\mu_0} \\
\vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots \\
\frac{dn(t = t_2)}{dpH_{\text{min}}} & \frac{dn(t = t_2)}{dpH_{\text{opt}}} & \frac{dn(t = t_2)}{dpH_{\text{max}}} & \frac{dn(t = t_3)}{du_{\text{opt}}} & \frac{dn(t = t_2)}{d\mu_{\text{opt}}} & \frac{dn(t = t_3)}{dn_0} & \frac{dn(t = t_3)}{d\mu_0} \\
\end{bmatrix}
\]  

(B.18)

For two experiments, considering that these are independent of each other, the partial derivatives of cell concentration of one experiment to the primary models of the other are equal to 0, i.e.:

\[
\frac{dn_1}{dn_{0,2}} = \frac{dn_2}{dn_{0,1}} = 0 \quad \text{(B.19)}
\]

\[
\frac{dn_1}{d\mu_{0,2}} = \frac{dn_2}{d\mu_{0,1}} = 0 \quad \text{(B.20)}
\]

\[
\frac{dn_1}{dn_{\text{max},2}} = \frac{dn_2}{dn_{\text{max},1}} = 0 \quad \text{(B.21)}
\]

In this way, the Jacobian matrix for a set of two experiments is given by:

\[
J_{1,2} = 
\begin{bmatrix}
\frac{dn_1(t = t_1)}{dpH_{\text{min}}} & \cdots & \frac{dn_1(t = t_1)}{dpH_{\text{opt}}} & \cdots & \frac{dn_1(t = t_1)}{dpH_{\text{max}}} & \frac{dn_1(t = t_2)}{d\mu_{\text{opt}}} & \cdots & \frac{dn_1(t = t_2)}{d\mu_0} & \frac{dn_1(t = t_2)}{dn_0,1} & \cdots & \frac{dn_1(t = t_2)}{dn_0,2} & 0 & 0 \\
\vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots \\
\frac{dn_2(t = t_1)}{dpH_{\text{min}}} & \cdots & \frac{dn_2(t = t_1)}{dpH_{\text{opt}}} & \cdots & \frac{dn_2(t = t_1)}{dpH_{\text{max}}} & \frac{dn_2(t = t_2)}{d\mu_{\text{opt}}} & \cdots & \frac{dn_2(t = t_2)}{d\mu_0} & \frac{dn_2(t = t_2)}{dn_0,1} & \cdots & \frac{dn_2(t = t_2)}{dn_0,2} & 0 & 0 \\
\vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots & \vdots \\
\frac{dn_2(t = t_2)}{dpH_{\text{min}}} & \cdots & \frac{dn_2(t = t_2)}{dpH_{\text{opt}}} & \cdots & \frac{dn_2(t = t_2)}{dpH_{\text{max}}} & \frac{dn_2(t = t_3)}{d\mu_{\text{opt}}} & \cdots & \frac{dn_2(t = t_3)}{d\mu_0} & \frac{dn_2(t = t_3)}{dn_0,1} & \cdots & \frac{dn_2(t = t_3)}{dn_0,2} & \frac{dn_2(t = t_3)}{dn_{\text{max},2}} & \frac{dn_2(t = t_3)}{dn_{\text{max},1}} \\
\end{bmatrix}
\]  

(B.22)

In the parameter estimation procedure, the Jacobian matrix is initially supplied to the \textit{lsqnonlin} routine of the \textit{Optimization Toolbox} of Matlab, along with a vector of residuals at each sampling time, reason why this vector has the same number of lines as the Jacobian matrix. For the example with two experiments:
The value of the residuals included in this vector is then given by the difference between the natural logarithm of the measured cell density, $n_m$, and the model prediction, $n_p$, at the sampling time $t$ of each experiment. In the secondary modelling, the `lsqnonlin` routine converges to the optimal combination of primary and secondary model parameters by minimizing the sum of squared errors from the residuals vector. In turn, the (local) minimum of the sum of squared errors is obtained by identifying the correspondent values of the primary and secondary model parameters partial derivatives (and consequently, of the partial derivatives in the Jacobian matrix). Initially, the Jacobian matrix is computed for an initial guess for the undetermined model parameters, $p = p^0$. Each iteration, $p^0$ is replaced by new parameter estimates (therefore, changing the sensitivity of the model output to those parameters), until convergence is attained.