Impact of pH on the cardinal temperatures of E. coli K12: Evaluation of the gamma hypothesis

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Accurate description of the effect of (a combination of) environmental conditions on the microbial growth rate is of high importance for the predictive quality of models used in predictive microbiology. According to the previously defined gamma hypothesis, environmental factors act independently on the microbial growth dynamics. For temperature specifically, this concept implies that the cardinal temperatures \( T_{\text{min}}, T_{\text{opt}}, T_{\text{max}} \) are only determined by the microbial temperature response and not influenced by other environmental conditions. In this research, it is evaluated if pH affects the values of the cardinal temperatures. Hereto, the parameters of the Cardinal Temperature Model with Inflection (CTMI, Rosso, Lobry, & Flandrois, 1993) have been derived from an extended experimental data set. \( T_{\text{min}} \) and \( T_{\text{max}} \) as a function of pH seem to follow a parabolic trend, which is in contradiction to the gamma hypothesis: the relation implies that pH does affect the cardinal temperatures. In contrast, the experimental data possibly show that the \( T_{\text{max}} \) value is approximately constant at moderate pH values. This observation partially validates the gamma hypothesis. For \( T_{\text{opt}} \), no obvious trend could be observed. Independently of the exact relation, a combination of a pH stress and extreme temperature act synergistically on the microbial growth dynamics.

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1. Introduction

When assessing microbial food safety and quality, a highly important factor is the evaluation of the effect of processing and storage conditions on the microbial dynamics. Predictive microbiology is a useful tool for the (food) industry to quantify microbiological risks related to certain processing techniques and/or subsequent food storage and distribution.

Secondary predictive models are being developed to describe the influence of (changing) environmental conditions on growth and/or inactivation dynamics. Models mainly describe the effect of the environment on the microbial growth or inactivation rate. Secondary models developed in the early stages of predictive microbiology mainly focused on the effect of temperature, and to a lesser extent on pH and water activity. Afterwards, square root- and cardinal parameter-type models were constructed considering multiple environmental factors, based on the gamma concept. In this concept, it is assumed that different influencing factors act independently such that they can be used in a multiplicative way in the secondary models. More specifically, it implies that the value of typical model parameters \( (T_{\text{min}}, a_{\text{w,\min}}, \text{etc.}) \) does not change when other environmental factors become more stressing. Towards the beginning of this century, more complex secondary growth rate models, i.e., models containing more than three factors, were developed. For instance, Devlieghere et al. (2000) developed a model that includes temperature, water activity, \( \text{CO}_2 \), and sodium lactate to describe the dynamics of the spoilage organism \( \text{Lactobacillus sake} \). The model constructed by Ross, Ratkowsky, Mellefont, and McMeekin (2003) for \( \text{Escherichia coli} \) includes temperature, water activity, pH, and lactic acid.

The above models do not include possible interactions between the considered environmental conditions. In the last decades, discussion has emerged with respect to the validity of the gamma concept. According to Bidlas and Lambert (2008), the gamma hypothesis is a valid approach to describe the effect of multiple environmental factors on the microbial growth rate. For \( \text{E. coli} \), for instance, they showed that Na-acetate and K-sorbate work...
independently at pH values between 5.5 and 6.5. However, many researchers have observed that synergistic effects exist between (certain) environmental factors. In such case, different intrinsic or extrinsic properties of the food (model system) no longer act independently but rather synergistically. Most often, synergistic interactions are observed when approaching the growth/no growth boundary.

Starting from the observations of synergistic interactions, model construction factors have been constructed that take into account the interactions between influencing factors. Augustin and Carlier (2000b) adapted a multiplicative secondary model previously published to describe independently the effects of environmental factors on the growth rate of Listeria monocytogenes (Augustin & Carlier, 2000) by including interactions between the environmental factors. To enable accurate description of the growth of Listeria innocua as a function of temperature, pH, and organic acids, Le Marc et al. (2002) proposed a model expansion that includes the interaction between these factors in the neighbourhood of the growth limits. Coroller et al. (2005) constructed model correction factors that enable to quantify the synergistic effect of different weak acids. Here, model factors were added to the cardinal parameter model with interactions as developed by Le Marc et al. (2002).

In the presented work, the effect of temperature and pH on microbial growth, and more specifically their (possible) interaction, is studied in detail. In particular, this study investigates whether the environmental pH affects the cardinal temperatures \(T_{\text{min}}, T_{\text{opt}}, T_{\text{max}}\) of E. coli K12 MG1655. During the previous decades, both interactive effects and independent relations have been observed. Many studies that focus on the growth/no growth region observe that interactions between these two factors exist at highly stressing temperature and/or pH conditions (see, e.g., Presser, Ross, & Ratkowski, 1998; Valero, Carrasco, Pérez-Rodriguez, García-Gimeno, & Zurera, 2006). On the other hand, many models have been developed and validated that consider an independent relation (see, e.g., Ross et al., 2003; Wijtzes, de Wit, Huis in’t Veld, van’t Riet, & Zwietering, 1995). However, when studying these validation exercises in more detail, it can be observed that environmental regions taken are mainly situated in (sub)optimal conditions, far from highly stressing conditions. Taking into account all this knowledge, it can be expected that some kind of interaction exists between temperature and pH with relation to microbial growth.

The focus of this manuscript is on the identification of the specific nature of this synergistic relation between environmental temperature and pH. Here, dynamic temperature experiments are performed at constant pH values in a computer-controlled bioreactor to guarantee accurate control of both temperature and pH. A profound study is performed, i.e., a high number of pH levels is considered to cover the total region that permits growth. For non-stressing pH levels, one dynamic experiment was selected. For (highly) stressing pH conditions, at least two dynamic experiments are combined to assure sufficient experimental information. From these experimental data, cardinal temperatures, cardinal pH values, and possible interactions, are determined.

The results presented in this manuscript are an extension of the work presented at ICPMF7, which included a preliminary discussion about the effect of pH on the cardinal temperatures (Baka, Van Derlinden, Boons, & Van Impe, 2011).

2. Materials and methods

2.1. Experimental set-up

2.1.1. Bacterial strain

E. coli K12 MG1655 (CGSC#6300) was acquired from the E. coli Genetic Stock Center from Yale University. A stock culture was stored at \(-80\) °C in Brain Heart Infusion broth (BHI) (Oxoid), supplemented with 2.5% glycerol (Acros Organics).

2.1.2. Inoculum preparation

For the preparation of the inoculum, a loopful of the stock culture was inoculated into a 50 mL Erlenmeyer containing 20 mL BHI, which was incubated in a controlled incubator at 37 °C (model KPB6151, Termaks). After 9 h, 20 μL was transferred into 20 mL fresh BHI and again incubated at 37 °C for 15 h.

2.1.3. Experimental design

Dynamic experiments, i.e., with varying temperature, were implemented at different, constant pH values, selected within \([4.5, 5.5, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5] \) pH. Details for all profiles are given in Table 1. Three temperature regions for experimental data collection were selected, i.e., \([45 \degree C, 50 \degree C, 55 \degree C, 60 \degree C, 65 \degree C, 70 \degree C, 75 \degree C, 80 \degree C] \) and \([35 \degree C, 30 \degree C, 25 \degree C, 20 \degree C, 15 \degree C, 10 \degree C, 5 \degree C, 0 \degree C]\). Profiles for experiments from \(45 \degree C\) temperature remained constant at \(45 \degree C\) for 2.5 h. When started from \(43 \degree C\) to \(35 \degree C\), temperature remained constant for 4 h and 2 h, respectively. This constant phase was followed by a temperature decrease at the rate given in Table 1. When the lower boundary was reached, temperature remained constant for more than 35 h.

For all experiments, the temperature change rate is smaller than \(5 \degree C/h\) as previous experiments showed that higher rates can induce an intermediate lag phase (see Van Derlinden, Bernaerts, & Van Impe, 2008).

The first temperature region was selected based on optimal experiments designed by Van Derlinden, Bernaerts, and Van Impe (2010). Profiles in the two remaining regions were mainly implemented for stressing pH values where insufficient information could be collected from a single experiment.

2.2. Bioreactor experiments

Dynamic experiments were performed in a bench top bioreactor (BioFlo 3000, New Brunswick Scientific Inc.).

Table 1

<table>
<thead>
<tr>
<th>pH</th>
<th>Temperature profiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>([45 \degree C, 50 \degree C, 55 \degree C, 60 \degree C, 65 \degree C, 70 \degree C, 75 \degree C, 80 \degree C] )</td>
</tr>
<tr>
<td>5.5</td>
<td>([43 \degree C, 27 \degree C, 22 \degree C, 17 \degree C, 12 \degree C, 7 \degree C, 2 \degree C, -1 \degree C])</td>
</tr>
<tr>
<td>6.5</td>
<td>([42 \degree C, 21 \degree C, 10 \degree C, -1 \degree C])</td>
</tr>
<tr>
<td>7.75</td>
<td>([41 \degree C, 20 \degree C, 10 \degree C, -1 \degree C])</td>
</tr>
<tr>
<td>8.75</td>
<td>([40 \degree C, 19 \degree C, 10 \degree C, -1 \degree C])</td>
</tr>
<tr>
<td>9.75</td>
<td>([39 \degree C, 18 \degree C, 10 \degree C, -1 \degree C])</td>
</tr>
</tbody>
</table>

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filled with 3.5 L BHI, was aerated at 2 L/min and the stirrer speed was set at 400 rpm. To avoid accumulation of foam, 1 mL of antifoam agent (Sigma) was added at the start of the experiment.

Time—temperature profiles were implemented via the AF5-biocommand Software (New Brunswick Scientific Inc.). The bioreactor unit was connected to a circulation cooler (CFT-33, Neslab Instruments Inc.) enabling temperature control below room temperature. The pH value was kept constant by the addition of base (1 N KOH) (Acros Organics) or acid (1 N H₂SO₄) (Acros Organics).

### 2.3. Mathematical modelling

#### 2.3.1. Primary model: model of Baranyi and Roberts (1994)

To describe the growth of *E. coli* K12 as a function of time, the primary growth model of Baranyi and Roberts (1994) is applied

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

\[
\frac{dQ(t)}{dt} = \mu_{\text{max}} \cdot Q(t)
\]

with \(N(t)\) [CFU/ml] the cell density at time \(t\), \(N_{\text{max}}\) the maximum value for \(N(t)\), \(Q(t)\) [ ] the physiological state of the cells, and \(\mu_{\text{max}}\) [1/h] the maximum specific growth rate. To describe the effect of temperature and/or pH on the growth rate, the Baranyi model is combined with the CTMI and/or CPM model, respectively (see below).

#### 2.3.2. Gamma concept

The gamma concept is based on the assumption that the effect of environmental factors on the growth rate of microorganisms can be combined by multiplying the separate effects (Rosso, Lobry, Bajard, & Flandrois, 1995; Zwietering, de Wit, & Notermans, 1996). For the specific case studied in this work, this can be expressed as follows:

\[
\mu_{\text{max}}(T, \text{pH}) = \mu_{\text{opt}} \cdot \gamma(T) \cdot \gamma(\text{pH})
\]

where \(\gamma(T)\) and \(\gamma(\text{pH})\) are described by Equations (3) and (4), respectively.

#### 2.3.3. Cardinal temperature model with inflection (CTMI)

The Cardinal Temperature Model with Inflection relates the maximum specific growth rate with temperature \((T \text{ [C]}\) (Rosso et al., 1995):

\[
\mu_{\text{max}} = \begin{cases} 
T < T_{\text{min}}, & 0 \cdot 0 \\
T_{\text{min}} < T < T_{\text{opt}}, & \mu_{\text{opt}} \times \gamma(T) \\
T > T_{\text{opt}}, & 0 \cdot 0 
\end{cases}
\]

with \(T_{\text{min}} \text{ [C]}\) and \(T_{\text{max}} \text{ [C]}\) the temperature at which growth is no longer observed, and \(T_{\text{opt}} \text{ [C]}\) the temperature at which the maximum specific growth rate equals its optimal value \(\mu_{\text{opt}} [1/h]\).

#### 2.3.4. Cardinal pH model (CPM)

The CPM describes the effect of pH on the maximum specific growth rate (Rosso et al., 1995), i.e.,

\[
\mu_{\text{max}}(\text{pH}) = \mu_{\text{opt}} \cdot \frac{(\text{pH} - \text{pH}_{\text{max}})(\text{pH} - \text{pH}_{\text{min}})}{(\text{pH} - \text{pH}_{\text{min}})(\text{pH} - \text{pH}_{\text{max}}) - (\text{pH} - \text{pH}_{\text{opt}})^2} = \mu_{\text{opt}} \cdot \gamma(\text{pH})
\]

with \(\text{pH}_{\text{min}}, \text{pH}_{\text{opt}}, \text{pH}_{\text{max}}\) the cardinal pH values, and \(\mu_{\text{opt}} [1/h]\) the growth rate at the optimal pH. At pH values below \(\text{pH}_{\text{min}}\) or above \(\text{pH}_{\text{max}}\), the growth rate \(\mu_{\text{max}}\) is equal to zero.

#### 2.4. Parameter estimation

Model parameters were estimated from the set of experimental data via the minimization of the sum of squared errors, using the *lsqnonlin* routine of the *Optimization Toolbox* of Matlab version R2010b (The MathWorks Inc.). For parameter estimation based on one experiment (with \(M\) measurements), the SSE is calculated as follows:

\[
\text{SSE} = \sum_{i=1}^{M} (n(t_i) - n(t_i, \text{p}))^2
\]

with \(n(t_i)\) the cell density measurement and \(n(t_i, \text{p})\) the model prediction at time \(t_i\). The vector \(\text{p}\) contains the considered parameters of the primary and secondary model. For the global parameter estimation, the SSE based on \(N\) experiments (with \(M_N\) measurements) is defined as follows.

\[
\text{SSE} = \sum_{j=1}^{N} \sum_{i=1}^{M_N} (n_j(t_i) - n_j(t_i, \text{p}))^2
\]

### 3. Results

#### 3.1. Experimental data

For most experiments, typical growth curves are obtained, i.e., a (short) lag phase is followed by an exponential growth phase and
a stationary phase (data not shown). Only at pH values 4.5, 9 and 9.5, cell density data as a function of time do not follow this typical trend (see Fig. 1).

For the case of pH 4.5, two experiments were performed with different initial inoculum levels, i.e., 7 and 13 ln(CFU/mL). In both cases, the cells were added to the medium a few hours before the first sample is taken. During these hours, it seems that inactivation takes place as the first cell counts are significantly lower than the 7 and 13 ln(CFU/mL) that were initially added. For the lower inoculum level, no colonies appeared on the BHI plates and no further growth was observed. In the case of the higher inoculum level, an initial inactivation phase is followed by a (minor) growth phase. For pH 9, three dynamic experiments were performed (see Table 1). The experiment within the temperature region [45 °C, 15.1 °C] yielded an initial logarithmic growth phase, followed by a decrease in cell number. When the final constant temperature of 15.1 °C was attained, the cell number remained stable (Fig. 1). For pH 9.5, growth of E. coli was observed for approximately 5 h. Hereafter, inactivation is induced and no viable cells could be observed after 8 h.

The above described a-typical growth curves, i.e., the combinations of growth and inactivation, are most likely due to the combination of the highly stressing low/high pH and/or low/high temperature. Therefore, these three data sets were finally excluded from further data processing.

In a first approach, all remaining data sets, i.e., for all pH values, were combined in a global fit to identify the seven parameters of the combined CTMI and CPM model, i.e., the three cardinal temperatures, the three cardinal pH values and the optimal growth rate. In the second approach, CTMI parameters (cardinal temperatures and $\mu_{\text{opt}}$) were estimated for each pH value separately.

### 3.2. Global cardinal temperatures and pH estimation

In this first approach, all data were combined in a global fitting procedure in which both the parameters of the CTMI and the CPM were identified (7 parameters). The obtained model parameter values are listed in Table 2, as well as the corresponding standard errors. Standard errors for the cardinal temperature models are ranging from 2% to 12.5%. Even more extreme estimation errors are obtained for $\mu_{\text{opt}}$ and the cardinal pH values.

### 3.3. Estimation of cardinal temperatures as a function of pH

In this second approach, $T_{\text{min}}, T_{\text{opt}}, T_{\text{max}}$ and $\mu_{\text{opt}}$ values were determined for each pH value by fitting the experiment(s) with a combination of the Baranyi model (1994) and the CTMI model. In cases where multiple temperature profiles were implemented for one pH value, all data sets (i.e., obtained from the two or three temperature profiles) were combined in a global fitting procedure. Generally, experimental data description with the combination of the growth model of Baranyi and Roberts (1994) and the CTMI model is acceptable (data not shown). Fig. 2 depicts the estimated values for the cardinal temperatures and $\mu_{\text{opt}}$ as a function of pH, together with the standard errors (error bars). These figures enable to determine the relation between the environmental pH and the cardinal temperatures, and as such, to evaluate the gamma hypothesis. $T_{\text{min}}$ as a function of pH follows a quadratic trend. The highest values for $T_{\text{min}}$ are obtained

![Fig. 1. Growth curves of Escherichia coli K12 at stressing pH values: (upper) pH = 4.5, (centre) pH = 9, (lower) pH = 9.5, for the dynamic temperature profile from 45 °C to 15.1 °C (see Table 1).](image-url)

### Table 2

| Parameter estimates and standard errors resulting from the global fit. |
|-----------------|-------------|-----------------|
| Parameter       | Estimated value | Standard error  |
| $T_{\text{min}}$ | 9.23 °C      | 1.21            |
| $T_{\text{opt}}$| 35.46 °C      | 1.51            |
| $T_{\text{max}}$| 45.77 °C      | 0.71            |
| $\mu_{\text{opt}}$ | 2.52 h⁻¹ | 10.27          |
| pH_{min}        | 3.95         | 23.81           |
| pH_{opt}        | 6.16         | 18.63           |
| pH_{max}        | 9.71         | 51.45           |
at (close to) optimal pH values. When pH becomes more stressing, *E. coli* is able to grow at even lower temperatures, i.e., the $T_{\text{min}}$ values decrease with the stress level. Another phenomenon appears for $T_{\text{max}}$ and $\mu_{\text{opt}}$ as a function of pH. For these parameters, a higher pH stress results in a decreased growth potential of *E. coli*, i.e., both $T_{\text{max}}$ and $\mu_{\text{opt}}$ are lower for more stressing pH values. In contrast, a second interpretation of the relation between $T_{\text{max}}$ and $\mu_{\text{opt}}$ and pH can be suggested. In the range of pH 6–8.5 and 6–9 respectively, $T_{\text{max}}$ and $\mu_{\text{opt}}$ seem to be approximately constant. Out of the boundaries of the considered areas, their values decline. So, for non-stressing pH values, there is no interaction between the two environmental factors ($T$ and pH), and $\mu_{\text{opt}}$ is not affected. For $T_{\text{opt}}$ as a function of pH no clear trend can be observed.

4. Discussion

The purpose of this research was to examine the combined effect of temperature and pH on the growth dynamics of *E. coli* K12 and evaluate the synergistic effect between the selected environmental conditions. The CTMI and CPM model were used to describe the experimental data. In a first step, one set of cardinal temperatures and pH values are defined to describe all experimental data. Next, the cardinal temperatures as a function of pH are determined for *E. coli*. Hereto, experimental data obtained from dynamic temperature experiments performed at a series of constant pH values, were analysed.

4.1. Selection of temperature regions

Dynamic experiments were selected as these are considered more realistic and more informative than a limited set of experiments at constant temperatures (Van Derlinden et al., 2008). A previous implementation of optimal experiment design showed that rapid and large temperature changes are preferred to slow and small changes for the accurate estimation of the CTMI parameters (Bernaerts, Versyck, & Van Impe, 2000; Van Derlinden et al., 2008). In Van Derlinden et al. (2010), a single experiment was designed using optimal experiment design for parameter estimation to optimize the estimation of the four CTMI parameters. This optimal experiment was used as the starting point for the experiments implemented at non-stressing pH values. However, at pH values close to the minimum and maximum pH for growth, a single optimal experiment proved to yield insufficient information: the lag phase was too long or the duration of the exponential phase was too short (data not shown). This is probably due to the combination of stressing levels for both temperature (i.e., 45 °C) and pH (i.e., values close the minimum and maximum values that permit growth). As a result, only a very low number of data points were obtained at these conditions and additional temperature profiles were applied (see Table 1).

4.2. Evaluation of the overall cardinal values

Starting from these dynamic experiments, it is evaluated if using one set of cardinal temperatures and pH values can yield an acceptable description of the *E. coli* growth dynamics. Next to the quality of the model description, the accuracy of the obtained cardinal values is checked, i.e., the values are compared with values reported by other researchers.

The combination of the growth model of Baranyi and Roberts and the CTMI model is not able to describe the growth curves under dynamic temperature conditions and at different pH values accurately. At moderate pH values, the overall trends in the growth
curves are followed. However, much larger deviations between cell counts and model description are observed at the extremes. In addition, this global fitting procedure does not yield accurate reliable values for the parameter estimation, i.e., the values of some cardinal parameters seem rather unrealistic and the errors are unacceptably high (Table 2).

4.2.1. Cardinal temperatures

For E. coli K12, the cardinal temperatures are estimated as follows: $T_{\text{min}} = 11.2 \, ^\circ \text{C}$, $T_{\text{opt}} = 41 \, ^\circ \text{C}$ and $T_{\text{max}} = 48 \, ^\circ \text{C}$ (Rosso, Lobry, & Flandriens, 1993). Also for the same strain, Van Derlinden et al. (2008) determined the cardinal temperatures, based on dynamic bioreactors experiments at 9.14 °C, 39.6 °C, and 46.7 °C for $T_{\text{min}}$, $T_{\text{opt}}$ and $T_{\text{max}}$, respectively. Based on these references, it can be stated that the optimum growth temperature is significantly underestimated. The value of 35.5 °C is more than 4 °C lower than values generally reported. The value for $T_{\text{min}}$ seems acceptable. However, the $T_{\text{max}}$ value found in the presented work might be an underestimation as it is at least one degree lower than reported values.

Cardinal temperatures have also been defined for other E. coli strains. Rosso et al. (1995) defined the cardinal temperatures for E. coli O157:H7 at 3.06 °C, 41.10 °C, and 45.06 °C, for $T_{\text{min}}$, $T_{\text{opt}}$ and $T_{\text{max}}$, respectively. Pinon et al. (2004) determined the cardinal temperatures for the E. coli O26 strain in cooked poultry meat: $T_{\text{min}} = 6.81 \, ^\circ \text{C}$, $T_{\text{opt}} = 40.97 \, ^\circ \text{C}$, $T_{\text{max}} = 45.16 \, ^\circ \text{C}$ (Membrè et al., 2005) identified the following cardinal growth temperatures: $T_{\text{min}} = 4.9 \, ^\circ \text{C}$, $T_{\text{opt}} = 41.1 \, ^\circ \text{C}$ and $T_{\text{max}} = 45.8 \, ^\circ \text{C}$ for 10 strains of E. coli isolated from meat products. Again, significantly higher values for $T_{\text{opt}}$ are obtained. Compared to the values for E. coli K12, the minimum growth temperatures for these (pathogenic) strains are lower. Values for $T_{\text{max}}$ are close to the values found for E. coli K12.

4.2.2. Cardinal pH values

Evaluation of the cardinal pH values is more difficult. For the specific strain E. coli K12, little research has been performed to define its minimum and maximum pH for growth. Furthermore, it is generally known that not only pH but also the type of acid(s) determines the microbial growth boundaries. For example, for E. coli K12, growth is observed at a pH of 5.9. In contrast, a low concentration of acetic acid (20 mM) prevented the outgrowth at the same pH value (Diez-Gonzalez & Russell, 1997).

From a series of preliminary experiments in which E. coli K12 is grown at a constant pH value at 40 °C, it can be observed that the lowest pH yielding growth was 4.5 (data not shown). At the other side of the growth region, the highest pH value for which growth was observed was 8.5. From these experiments it seemed, however, that the optimal pH for E. coli K12 is around 7–7.5, which is significantly higher than the value obtained from the global description of the presented dynamic experiments. In addition, the experiments in Fig. 1 show that growth at pH 4.5 and 9.5 is not really supported. From the observation of a-typical growth experiments in Fig. 1 show that growth at pH 4.5 and 9.5 is not really supported. From the observation of a-typical growth

4.3. Interaction between temperature and pH: evaluation of the gamma concept

Based on the above observations, it can be stated that one set of cardinal temperatures and pH values cannot describe all data accurately. As such, the gamma concept cannot be validated and some kind of interaction exists between temperature and pH.

Fig. 2 shows that, for some parameters of the CTMI, a specific relation seems to exist, i.e., $T_{\text{min}}$ and $T_{\text{max}}$ as a function of pH follow a parabolic relation. The two environmental factors, i.e., pH and temperature, interact with each other. For stressing pH values, $T_{\text{min}}$ and $T_{\text{max}}$ values are lower than for more optimal pH values. For $\mu_{\text{opt}}$, a similar trend can be observed. This is rather straightforward as it is generally known that the growth rate depends on the environmental pH and that the maximum of the $\mu_{\text{opt}}$ (pH) relation corresponds with the optimal pH value. No specific relation can be observed for the optimum temperature for growth. It can be summarized that the boundaries of the temperature region supporting growth, i.e., $T_{\text{min}}$ and $T_{\text{max}}$, are not independent from the second environmental factor (pH).

These relations for $T_{\text{min}}$ and $T_{\text{max}}$ as a function of pH are in contrast to the observations of Wijtzes et al. (1995) and Lambert and Bidlas (2007a, 2007b), whose experimental data supported the gamma concept. Wijtzes et al. (1995) found that $T_{\text{min}}$ as a function of pH could be supposed constant and around zero for Lactobacillus curvatus. Furthermore, Lambert and Bidlas (2007b) found that the effect of pH is also independent of temperature for Enterobacter sakazakii.

Next to the suggested parabolic relation, $T_{\text{max}}$ and $\mu_{\text{opt}}$ as a function of pH can be considered constant at optimum pH values ($\approx 6.5$–$8.5$). This observation corresponds to the adapted definition of the gamma hypothesis, i.e., no interaction exists at moderate conditions and synergies emerge when approaching the growth boundaries. This relation for $T_{\text{max}}$ is similar to what Le Marc et al. (2002) observed, i.e., for the main part of the growth range the assumption of independent effects of temperature and pH is sufficient to describe the bacterial growth. Effects are only synergetic at very stringent conditions.

Independently of the hypothesis supported, it is remarkable that the same trend is observed for $T_{\text{min}}$ and $T_{\text{max}}$, i.e., lower cardinal temperature values correspond to high and low pH values. This implies that the interaction between temperature stress and pH stress works different at low temperatures and at high temperatures.

For $T_{\text{max}}$, the observed relation is in accordance with the hurdle theory (Leistner, 1995): $T_{\text{max}}$ decreases when combined with stressing pH. A similar, and expected, relation is observed for the growth rate, that is, $\mu_{\text{opt}}$ is higher in optimum pH values and lower in stressing ones.

On the other hand, $T_{\text{min}}$ seems to decrease slightly when combined with stressing pH values. This observation is contradictory to the hurdle theory: at low pH values, E. coli is able to grow at a lower temperature. Yet, microbial adaptation phenomena can be addressed to explain this observation. The ability of a stress-adapted microorganism to resist when it is exposed to another environmental stress is known as cross protection (Juneja & Novak, 2003). When bacterial cells are exposed to an environmental stress, they respond in several ways. Most likely they produce proteins, known as shock proteins, and their main function is to repair the damages caused by the stress factor or eliminate the stress agent (Ohtsuka, Kawashima, & Asai, 2007; Yousef & Courtney, 2003). In addition to a specific stress response (e.g., cold shock response or acid stress response), E. coli also has a general stress response, controlled by the sigma factor RpoS, which relates to more global changes in the cellular metabolism. According to Battesti, Majdalani, and Gottesman (2011), this general stress resistance is responsible for cross protection. Cheville, Arnold, Buchrieser, Cheng, and Kaspar (1996) also showed that the absence of RpoS significantly reduced resistance towards acid, salt, and heat, and the acid tolerance response was not induced. According to Ait-Ouazzou, Manas, Condon, Pagan, and Garcia-Gonzalo (2012), the RpoS is extremely important for the heat resistance of E. coli strains and triggering the RpoS via other environmental stresses will also improve the heat resistance.

With respect to the cross-protective effect of acid adaptation on temperature stress resistance, most research has focused on
the growth, survival and/or inactivation under heat stress. All research shows that an adaptation to pH stress, ranging from mild to stringent acidic conditions, increases the microbial heat resistance. Examples of higher heat resistance of E. coli strains as a result of the cross-protective effect of low pH values can be found in Buchanan and Edelson (1999), Velliu et al. (2011), Duffy et al. (2000) and Singh, Mullins, Simpson, and Dickson (2010).

When extrapolating this knowledge towards the maximum growth temperature, it would be expected that exposure to mild pH stress results in an increased $T_{\text{max}}$ value. Fig. 2, however, shows that this assumption is not valid for our case study considered. The highest $T_{\text{max}}$ values are observed for approx. neutral pH and the maximum growth temperature decreases when increasing or decreasing the pH.

Up to now, less attention has been paid to the relation between acid adaptation and microbial dynamics at low temperatures, highly relevant for cold-stored products. In 2003, Blank et al. showed that the protective response of two E. coli strains towards low temperatures also yields protection to low pH values (Blank, Cho, & Ismond, 2003). A cold shock did not alter the growth dynamics of E. coli at low pH values obtained by the addition of different organic acids. A clear effect, however, was observed for the survival of E. coli at pH values that do not support growth, i.e., under some conditions improved survival can be observed after a cold shock. A similar observation was made by Uyttendaele, Taverniers, and Debevere (2001), i.e., E. coli O157:H7 survives much better in TSB at pH 4.5 when incubated at 7 °C compared to incubation at 22 °C.

Xu, Lee, and Ahn (2008) studied the resistance of Salmonella enterica towards severe acid stress and cold environment after exposure to acid. Their experiments show that more Salmonella cells can survive in pH 4.0 at 4 °C after exposure to a pH of 5.0 for 2 or 7 h. In addition, cells (pre-adapted and non-adapted to low pH) are more sensitive to a pH of 4.0 at 20 °C compared to 4 °C. As such, the combination of low pH and low temperature yields a higher survival. Growth at 4 °C in a pH of around 7.0 was clearly faster for acid-adapted cells than for non-adapted cells.

Whereas these references report on the interaction between low pH and low temperature, the synergy between temperature and pH observed in our experiments is limited at a low pH but more clear at high pH values. At pH values between 5 and 7, which are the most relevant for food products, $T_{\text{min}}$ does not differ a lot. $T_{\text{min}}$ values range from approx. 10 °C at pH 5 to approx. 12 °C at pH 6.5. This observation indicates that assuming a constant pH value for food products with a pH ranging between 5 and 7 only results in small inaccuracies. However, following the trend when going from pH 7.0 to pH 5.0, it can be expected that a lower $T_{\text{min}}$ is obtained at pH 4.0, which corresponds with the above discussion.

In the domain of (food) microbiology, a myriad of studies exists that investigate the interactions among different environmental factors, specifically under stressing conditions. The collection of available research clearly indicates that synergistic interactions highly depend on the total physico-chemical conditions, but also on the physiological cell state and the microbial strains considered. Therefore, it has to be evaluated if the results presented in the manuscript are transferable to other E. coli strains or species, or other conditions. Moreover, it is likely that the relation between the environmental pH and the cardinal temperatures depends on the acid that defines this pH. In our work, a strong acid was applied to set the pH value. When using an organic, weak acid, an additional antimicrobial effect emerges from the undissociated acid. This additional stress might change the relation between cardinal temperatures and pH significantly.

5. Conclusions

The aim of this research was the evaluation of the effect of the environmental pH on the growth dynamics of E. coli K12 under dynamic temperature conditions ranging from high to low temperatures. These dynamic experiments revealed that it is not possible to accurately describe all experimental growth curves using one set of cardinal temperatures and one set of cardinal pH values. As such, it can be stated that the gamma concept is not valid under the considered environmental conditions.

When defining cardinal temperatures for each pH value separately, a parabolic relation for $T_{\text{min}}$, $T_{\text{max}}$ and $\mu_{\text{opt}}$ as a function of pH is observed. No clear trend seems to exist for the $T_{\text{opt}}$—pH relation. A second interpretation can be made on the relation between $T_{\text{max}}$ and $\mu_{\text{opt}}$ with pH, that is, for non-stressing pH values, $T_{\text{max}}$ and $\mu_{\text{opt}}$ follow the gamma hypothesis but for stressing pH and temperature act synergetically.

Further experiments need to be implemented to determine more accurately the true relations between the cardinal temperatures and the pH for E. coli K12. The implementation of additional pH values (e.g., closer to pH$_{\text{min}}$ and pH$_{\text{max}}$) or dynamic pH profiles at constant temperatures or combinations of dynamic temperature and pH profiles will yield additional information.

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