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An interaction model for the combined effect of temperature, pH and water activity on the growth rate of *E. coli* K12

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1 ABSTRACT

2 Previous research has indicated that more complex model structures than the
3 commonly used gamma model are needed to obtain an accurate prediction of the effect
4 of multiple environmental conditions on the microbial growth rate. Due to the
5 complexity associated with the development of such model structures, it is
6 recommended that the model structure is compatible with a modular model building
7 method.

8 In this research, a gamma-interaction model was built to describe the combined
9 effect of temperature, pH and water activity on the microbial growth rate of *E. coli* K12
10 based on a dataset of 68 bioreactor experiments. This novel interaction model was
11 compared with the standard gamma model. The model structures were tested separately
12 for the combined effects of (i) temperature and pH, (ii) pH and water activity, (iii)
13 temperature and water activity and (iv) temperature, pH and water activity.

14 Based on the results of this research, it was concluded that models for the
15 combined effect of environmental conditions need to allow for sufficient flexibility for
16 the description of combined effects of environmental conditions to obtain accurate
17 model predictions. In the current study, this flexibility was successfully introduced by
18 using the gamma-interaction model. A cross-validation study also demonstrated that
19 the predictions of the interaction model are more robust with respect to the specific data
20 used than the gamma model. As such, the gamma-interaction model provides food
21 producers and food safety authorities with a more accurate and reliable tool for the
22 prediction of the microbial growth rate as a function of multiple environmental
23 conditions.

25 **Keywords:** Microbial growth rate, interaction model, *Escherichia coli*, model
26 building, predictive microbiology.

27

28 1 INTRODUCTION

29 One of the most common problem statements in predictive microbiology research
30 concerns the development of mathematical models that describe the effect of multiple
31 environmental conditions on the microbial growth rate. These models allow food
32 producers and food safety authorities to assess microbial growth in a wide variety of
33 food products and processing conditions. In general, models are preferred that (i)
34 contain biologically interpretable parameters, (ii) have the possibility to be extended
35 towards new environmental conditions, (iii) are parsimonious and (iv) provide a good
36 prediction quality (Geeraerd et al., 2004). Cardinal parameter models are commonly
37 used to describe the stress that the non-optimality of a single environmental condition
38 induces on a microorganism, by describing the effect that this condition has on the
39 microbial growth rate. These models use biologically interpretable parameters and are
40 most commonly applied for the effects of temperature and pH (Ross et al. 1993, Ross
41 et al. 1995). An additional advantage of these models is that they can also be used within
42 the gamma concept (Zwietering, et al. 1993). This concept allows the combination of
43 effects of environmental conditions on the microbial growth rate after studying these
44 effects independently of each other. This property is beneficial to reduce the
45 experimental work load of building a mathematical model as the total number of
46 experiments for E environmental conditions when obtaining data at Λ different levels is
47 just $E \cdot \Lambda$ for a gamma model where it would be Λ^E for a conventional full factorial
48 design. The gamma model also simplifies the design of mathematical models structures
49 that parsimoniously describe such complex effects. Moreover, the gamma model allows
50 for a *modular* modelling approach. This means that an existing mathematical model is
51 easily extended to include the effect of new environmental conditions by identifying
52 them separately and completing the model equations.

However, several studies have demonstrated that models build according to the gamma concept lack accuracy and adaptations have been proposed to improve the model fit (Augustin and Carlier, 2000; Le Marc et al., 2002). Recently, the gamma-interaction model was published by Akkermans et al. (2017). This model was compared with the gamma model, the interaction model of Augustin and Carlier (2000) and the interaction model of Le Marc et al. (2002) based on their ability to describe the combined effect of temperature and pH on the growth rate of *Escherichia coli* K12. The research established that it was necessary to include multiplicative interactions to accurately describe the combined effect of these conditions on the microbial growth rate. Moreover, this study found that the gamma-interaction model did not only outperform the gamma model but also the models of Augustin and Carlier (2000) and Le Marc et al. (2002) with respect to the model fit. The interested reader is referred to Akkermans et al. (2017) for more details on modelling the combined effect of environmental conditions on the microbial growth rate.

The mathematical formulation of the gamma-interaction model allows the inclusion of different types of effects and interactions between specific combinations of these effects. However, the theoretical ability of this model structure to describe the interactions between other environmental conditions was not verified. To this end, the current research deals with the extension of a mathematical model describing the effect of temperature and pH on the microbial growth rate by including the effect of water activity and all relevant multiplicative interactions. As such, the aim of this research is to further test and validate experimentally the ability of the gamma-interaction model to add flexibility to the more basic gamma model following a modular modelling methodology.

2 MATERIALS AND METHODS

2.1 Experimental methods

2.1.1 Microorganisms and inoculum preparation

E. coli K12 MG1655 (CGSC#6300) was obtained from the *E. coli* Genetic Stock Center at Yale University. A stock culture was stored at -80°C in Brain Heart Infusion broth (BHI, Oxoid), supplemented with 20% (w/v) glycerol (Acros Organics). The inoculum was prepared in a three step procedure: (i) a loopful of stock culture was spread onto a BHI agar plate (BHIA, BHI supplemented with 14 g/L technical agar nr. 3, Oxoid) and incubated overnight at 37°C. (ii) Then, a single colony was transferred into a 50 mL Erlenmeyer containing 20 mL BHI and stored at 37°C for 9 h. (iii) Finally, 20 µL of the stationary phase culture was inoculated into 20 mL fresh BHI and incubated at 37°C for 15 h before inoculation.

2.1.2 Bioreactor experiments

Experiments were performed in a set of bioreactors (BioStat B, Sartorius Stedim GmbH). The reactor vessels were filled with 3.5 L of BHI. Temperature was measured with a PT100 resistance temperature detector. A circulation chiller enabled temperature control below room temperature. pH measurement was performed with a gel-filled pH electrode (Hamilton Company) and corrected for temperature variations. pH was controlled by addition of acid (1 N H₂SO₄, Sigma-Aldrich) or base (1 N KOH, Thermo Fisher Scientific) by a PID controller. The bioreactor was aerated at 0.2 L/min after autoclaving and the oxygen concentration was controlled at the stabilised oxygen level during the experiment. The reactor content was stirred at 75 rpm with Rushton impellers. To avoid foaming, 500 µL of an antifoaming agent (Y-30 emulsion, Sigma-Aldrich) was added prior to every experiment.

2.1.3 Sampling and microbiological analysis

Depending on the specific experimental conditions, a sample was taken from the bioreactor with a frequency between twice every hour and once every two hours during daytime. The appropriate dilutions were made in BHI and 49.2 μL of sample was plated onto BHIA plates, in triplicate, using a spiral plater (Eddy Jet, IUL Instruments s.a.). These plates were incubated at 37°C for about 15 h and then colonies were counted to obtain viable cell numbers (CFU/mL). The average over the three plates was used as the measured cell density of a sample. Experiments lasted between 12 and 200 h, depending on the growth rate and contained between 9 and 56 samples. The growth curves used for parameter estimations included the lag, exponential and stationary phases of growth.

2.1.4 Water activity measurement

Water activity values were controlled by the addition of NaCl (Sigma-Aldrich) to the growth medium before autoclaving the reactor vessels. The resulting water activity values were determined using a Decagon AquaLab 4TE dew point water activity meter. For this measurements three 5 mL samples of each vessel were measures in triplicate and the average of these nine measurements was used as the water activity of an experiment. For all experiments in which no NaCl was added to the medium, the water activity was taken equal to that determined for an autoclaved BHI solution being 0.9949. The salt concentrations required to achieve the desired water activity values were calculated based on a calibration curve of the water activity as a function of salt concentrations added to BHI media. This calibration curve was represented by a simple linear relationship:

$$a_w = -0.0058 \cdot NaCl + 0.9931 \quad (1)$$

2.2 Experimental design

The experimental design is represented in Table 1. The full dataset is composed of 8 different groups, depending of the type of stress that is applied to the microorganisms. For this purpose, a stress is defined as one or more environmental conditions that differ from their optimum value. By organising the data in these groups, each group will contribute to a specific part of the mathematical model that is built in this publication (e.g., the optimal conditions contribute to the estimation of the optimal growth rate and the conditions with temperature and pH stress contribute to the characterisation of interactions between temperature and pH). The total dataset contains 63 distinct sets of experimental conditions and 68 experiments (due to five replicates). Datasets a, b, c and e were used in Akkermans et al. (2017) to model the combined effect of temperature and pH on the microbial growth rate. The specific experimental conditions were always chosen in an iterative procedure during the model development to select those conditions that were expected to yield the most useful information on the estimation of the model parameters (i.e., around the extremes of the sensitivity equations; Bernaerts et al., 2005) and the discrimination between the competing model structures (i.e., at the conditions that gave the largest difference between the predictions of the competing models). In the datasets for combined stresses, some experimental conditions may appear very similar. However, due to the changes in three environmental conditions the microbial response varies considerably.

2.3 Mathematical models

2.3.1 Primary model

To describe the evolution of the logarithm of the cell density n [$\ln(\text{CFU/mL})$] with time t [h], the widely used primary model of Baranyi and Roberts (1994) was implemented in the following form:

$$\frac{dn(t)}{dt} = \frac{1}{1+\exp(-q(t))} \cdot \mu_{\max}(T, \text{pH}, a_w) \cdot [1 - \exp(n(t) - n_{\max}(T, \text{pH}, a_w))] \quad (2)$$

$$\frac{dq(t)}{dt} = \mu_{\max}(T, \text{pH}, a_w)$$

with $\mu_{\max}(T, \text{pH}, a_w)$ [h^{-1}] the maximum specific growth rate and $n_{\max}(T, \text{pH}, a_w)$ [$\ln(\text{CFU/mL})$] the maximum cell density for a specific temperature (T [$^{\circ}\text{C}$]), pH [–] and water activity (a_w [–]). The dependence of $n_{\max}$ on the environmental conditions was not included in this research. $q(t)$ [–] is a measure for the physiological state of the cells and serves to describe the lag phase of the growth curve. The initial values of $n(t)$ and $q(t)$ are respectively n_0 and q_0 .

2.3.2 Gamma model

The first and undeniable principle behind the gamma concept is that the maximum specific growth rate $\mu_{\max}$ [h^{-1}] of a microorganism can always be expressed as the optimum growth rate μ_{opt} [h^{-1}] multiplied with a factor representing the reduction of the growth rate due to non-optimal conditions (gamma factor). Mathematically this is defined as:

$$\mu_{\max}(e) = \mu_{\text{opt}} \cdot \gamma(e) \quad (3)$$

In this equation e is the set of environmental conditions that influences the microbial growth rate and causes its reduction to a non-optimal growth rate. The second principle of the gamma concept is the hypothesis that the reduction of the microbial

growth rate due to the full set of environmental conditions results from the multiplication of the reduction of the growth rate due to the non-optimality of the individual environmental conditions. This hypothesis is formulated in the following equation:

$$\gamma(e) = \prod_{k=1}^{N_e} \gamma(e_k) \quad (4)$$

with N_e the total number of environmental conditions.

2.3.3 Gamma-interaction model

The gamma-interaction model was developed by Akkermans et al. (2017) to describe interactions between the independent effects of environmental conditions on the microbial growth rate. This model is an extension of the gamma model explained in the previous section and fits in a modular model building procedure. For the case study of the combined effect of temperature, pH and water activity on the microbial growth rate, the full gamma-interaction model is written as:

$$\mu_{\max}(T, pH, a_w) = \mu_{\text{opt}} \cdot \gamma(T, pH, a_w) = \mu_{\text{opt}} \cdot \gamma(T) \cdot \gamma(pH) \cdot \gamma(a_w) \cdot \gamma_i(T, pH, a_w) \quad (5)$$

$$\begin{aligned} \gamma_i(T, pH, a_w) = & [1 - (1 - \gamma(T)) \cdot (1 - \gamma(pH))]^{\beta_{T,pH}} \\ & \cdot [1 - (1 - \gamma(pH)) \cdot (1 - \gamma(a_w))]^{\beta_{pH,a_w}} \\ & \cdot [1 - (1 - \gamma(T)) \cdot (1 - \gamma(a_w))]^{\beta_{T,a_w}} \\ & \cdot [1 - (1 - \gamma(T)) \cdot (1 - \gamma(pH)) \cdot (1 - \gamma(a_w))]^{\beta_{T,pH,a_w}} \end{aligned}$$

This model contains the interaction parameters $\beta_{T,pH}$, β_{pH,a_w} , β_{T,a_w} and β_{T,pH,a_w} as additional parameters with respect to the simple gamma model. Interaction parameters take respectively positive or negative values in cases of synergistic or antagonistic multiplicative interactions.

2.3.4 Model for the effect of temperature

The model for the independent effect of temperature on the microbial growth rate was taken from Akkermans et al. (2017):

$$\gamma(T) = \frac{(T-T_{\min})^3 \cdot (T-T_{\max})^2}{(T_{\text{opt}}-T_{\min}) \cdot [(T_{\text{opt}}-T_{\min}) \cdot (T-T_{\text{opt}}) - (T_{\text{opt}}-T_{\max}) \cdot (T_{\text{opt}}+T_{\min}-2T)] \cdot [(T-T_{\min}) \cdot (T-T_{\max}) - (T-T_{\text{opt}})^2]} \quad (6)$$

The model parameters are the three cardinal temperatures: (i) the minimum temperature that allow growth $T_{\min}$, (ii) the optimum temperature T_{opt} and (iii) the maximum temperature for growth $T_{\max}$. This model structure was originally build based on the observation of Van Derlinden et al. (2012) that *E. coli* K12 has relatively high growth rates at low temperatures.

2.3.5 Model for the effect of pH

Based on the results of Akkermans et al. (2017), the aCPM model was chosen to describe the effect of pH on the microbial growth rate:

$$\gamma(\text{pH}) = \left(\frac{(\text{pH}-\text{pH}_{\min})^{\eta} \cdot (\text{pH}-\text{pH}_{\max})}{(\text{pH}-\text{pH}_{\min})^{\eta} \cdot (\text{pH}-\text{pH}_{\max}) - (\text{pH}-\text{pH}_{\text{opt}})^2} \right)^{1/\kappa} \quad (7)$$

In this model, the minimum pH, $\text{pH}_{\min}[-]$, and the maximum pH, $\text{pH}_{\max}[-]$, are the growth boundaries. $\text{pH}_{\text{opt}}[-]$ is the pH at which the gamma factor becomes equal to 1. The aCPM contains two shape parameters κ and η that take values equal to or larger than 1. The value of κ is higher for microorganisms that show an increased tolerance with respect to changes of the pH from its optimum value. η has a value larger than 1 for microorganisms with a higher tolerance to suboptimal than to superoptimal conditions. Based on the results of Akkermans et al. (2017), the values of κ and η are set equal to 3 and 2, respectively.

2.3.6 Models for the effect of water activity

Two models are considered to describe the independent effect of water activity on the microbial growth rate through a gamma factor. The first model is the gamma factor of Zwietering et al. (1993), which is formulated as:

$$\gamma_{a_w}(a_w) = \frac{a_w - a_{w,min}}{1 - a_{w,min}} \quad (8)$$

This equation represents a simple linear relationship between the water activity and the reduction of the growth rate. This gamma factor itself only contains a single model parameter being the theoretical minimum water activity that allows microbial growth $a_{w,min}$.

The second model is the Cardinal a_w Model (CAM, Augustin and Carlier, 2000), which was based on the equation of the Cardinal Temperature Model with Inflection (Rosso et al., 1993). The CAM is formulated as:

$$\gamma(a_w) = \frac{(a_w - a_{w,min})^2 \cdot (a_w - 1)}{(a_{w,min} - a_{w,opt}) \cdot ((a_{w,opt} - 1) \cdot (a_{w,min} - 2 \cdot a_w + a_{w,opt}) + (a_w - a_{w,opt}) \cdot (a_{w,min} - a_{w,opt}))} \quad (9)$$

In this model $a_{w,min}$ is the minimum water activity that allows growth and $a_{w,opt}$ is the optimum water activity.

2.4 Parameter estimation and confidence intervals

All secondary models were identified using a *one-step* parameter estimation (Jewel, 2012). As such, the secondary models were combined with the primary model and this was treated as a single model. The combined model is able to describe microbial growth as a function of time for various environmental conditions. The parameters of this combined model were estimated directly on the selected growth

curves. The estimated model parameters were (i) the secondary model parameters and a separate (ii) n_0 (iii) q_0 and (iv) $n_{\max}$ for every experimental growth curve. For visual evaluation of the mathematical models, figures are included that depict the comparison between the logarithms of the growth rates predicted by the secondary models and the growth rates determined by estimating the primary model parameters on a growth curve at a single set of environmental conditions. This visualization was chosen as the difference between the logarithms of the growth rates better reflects the objective of the one-step parameter estimation than the difference between the growth rates themselves (Akkermans et al., 2016).

The optimal combinations of parameters were calculated using the *lsqnonlin* routine of the Optimization Toolbox of Matlab version 7.14 (The Mathworks Inc.). This routine was always combined with a multi-start method that generated multiple sets of uniformly distributed random initial values of the parameters to be optimised. The objective function of the parameter estimation was the minimization of the sum of squared errors (SSE) for N_m measurements:

$$SSE = \sum_{k=1}^{N_m} \left(n_{m,k}(t_k) - n_{p,k}(t_k, p) \right)^2 \quad (10)$$

with $n_{m,k}(t_k)$ the logarithm of the measured cell density and $n_{p,k}(t_k, p)$ the logarithm of the predicted cell density for a set of parameter p at time t_k . The 95% confidence interval of every parameter p_k was calculated based on the Student's t -distribution (Van Impe et al., 2001):

$$\left[p_k \pm t_{0.975, N_m - N_p} \cdot \sqrt{s_{p_k}^2} \right] \quad (11)$$

where N_p is the number of parameters and consequently $N_m - N_p$ is the number of degrees of freedom. As a one-step parameter estimation was used, both the primary and secondary model parameters are included in N_p . $s_{p_k}^2$ is the variance on the

parameter p_k and is found on the main diagonal of the variance covariance matrix V , which is approximated as the inverse of the Fisher Information Matrix F (Walter and Pronzato, 1997):

$$s_{p,k}^2 = V(k, k) \quad (12)$$

$$V = F^{-1} \quad (13)$$

$$F = \frac{1}{MSE} \cdot J^T \cdot J \quad \text{with} \quad MSE = \frac{SSE}{N_m - N_p} \quad (14)$$

with J the Jacobian matrix of the residual vector (difference between the measured and predicted cell densities) and MSE the mean sum of squared errors. The MSE is used in this research as a measure for the quality of fit. By placing the degrees of freedom in the denominator of this measure, a trade off is made between flexibility and model complexity. Low MSE values represent a high quality of fit. Based on the parameter estimation of the primary model to each individual growth curve, an MSE value is calculated for each dataset describing stresses due to multiple environmental conditions. These MSE 's represents the absolute lowest value that could only be achieved if the secondary model gave an exact description of the relationship between environmental conditions and the microbial growth rate. Such an MSE will be referred to as the *minimum MSE* or MSE_{min} in the remainder of this article.

3 RESULTS AND DISCUSSION

Firstly, a mathematical model was chosen to describe the individual effect of water activity as controlled by the addition of NaCl on the microbial growth rate of *E. coli*. The models required to describe the individual effects of temperature and pH were selected based on the work of Akkermans et al. (2017). Secondly, three models were built for the combined effects of (i) temperature and pH, (ii) pH and water activity and (iii) temperature and water activity. Then, a model for the combined effect of temperature, pH and water activity is constructed and validated on the full dataset of 68 experiments. Finally, the robustness of this model was tested based on cross-validation.

The cardinal models estimated in this section represent the growth limits and the optimal conditions as parameters. It should be stressed that these parameters are in fact the theoretical growth limits and optimal conditions (McMeekin et al., 2013), which are only equal to the real values if the model describes the exact relationship between the environmental condition and the growth rate.

3.1 Modelling the effect of water activity

The first step in extending a model for the combined effect of temperature and pH to include the effect of water activity is the identification of a model for the individual effect of water activity on the growth rate. Two models were found in literature that fit into the gamma concept (i) the normalised gamma factor of Zwietering et al. (1993) and the (ii) CAM, which are explained in Section 2.3.6. These models were fitted to the datasets at (i) optimal conditions (a) and (ii) the conditions that were only stressing with respect to water activity (d). As such, the dataset contained a total of 7 growth curves at 6 distinct conditions. The parameter estimates with 95% confidence bounds are represented in Table 2 together with the MSE values for both models. Moreover, the

logarithm of the experimental and modelled growth rates are compared in Fig. 1. This figure and the MSE demonstrate that the gamma factor of Zwietering et al. (1993) provides a more accurate approximation of the experimental dataset. As this model also contains one less parameter than the CAM, it is significantly more parsimonious. Moreover, the model parameters of the gamma factor of Zwietering et al. (1993) were estimated with higher accuracy. Based on these results, the gamma factor of Zwietering et al. (1993) was chosen to describe the individual effect of water activity on the microbial growth rate for the remainder of this study.

3.2 Modelling the combined effect of temperature and pH

The mathematical model for the combined effect of temperature and pH is built in three steps. First of all, the gamma model for the effects of temperature and pH is fitted to the combined datasets of temperature stress and pH stress (dataset a-c, omitting the data of combined temperature and pH stress). This step is aimed at finding the secondary model parameters according to the independent effects. Ideally, these model parameters should be the same for a mathematical model that is identified based on data for the combined stress of temperature and pH. A significant change in these model parameters will most likely be due to a lack in suitability of the model structure that is used to describe the combined effect.

Secondly, the gamma model for the combined effect of temperature and pH is tested on the dataset with combined temperature and pH stress (datasets a, b, c and e). Finally, the mathematical model is extended with an interaction factor to describe the combined effect of temperature and pH on the growth rate and tested on the same dataset. Even though this interaction model was build and tested on the same dataset in Akkermans et al. (2017), the full mathematical model was not tested on the full dataset

in this publication. As their research was aimed at testing the qualities of the interaction factor itself, the parameters for the model components for the individual effects were fixed based on the datasets for separate temperature and pH stress.

The parameter estimation results are summarised in Table 3 and the fits of the gamma model and the gamma-interaction model to datasets a, b, c and e are illustrated in Fig. 2. The parameter estimation results show, both based on the MSE values and the visual comparison of the model fits, that the gamma-interaction model provides a significant improvement of the ability of the model to accurately describe the effect of temperature and pH on the measured microbial growth curves. This confirms the findings of Akkermans et al. (2017) where the same model was built by separately identifying (i) the temperature effect, (ii) the pH effect and (iii) their interactions. The values of the parameters pH_{opt} and μ_{opt} in the gamma model changed significantly when including data at combined temperature and pH stress. This demonstrates that this model structure is unsuitable as it is, and interactions have to be included.

The MSE value of the gamma-interaction model was still significantly higher than the minimum value possible to describe this effect (MSE_{min}). The ratio between the MSE and the MSE_{min} demonstrates that the gamma-interaction model does not yet describe the combined effect of temperature and pH as accurate as the gamma model describes the independent effects. Moreover, there appears to be some autocorrelation in the errors between measured and predicted growth rates in Fig. 2 (b). This indicates that some room for improvement exists in the full model structure that was used. It should be noted that this discrepancy may just as well be caused by the model structure of one of the independent effects as by the model structure used for the interactions.

3.3 Modelling the combined effect of pH and water activity

A model for the combined effect of pH and water activity was built in the same way as that for temperature and pH. First, the gamma model was tested on a dataset that did not contain any experimental conditions that were stressing with respect to both pH and water activity. Then, both the gamma model and the gamma-interaction model were tested on a dataset that contained experimental conditions that were stressing with respect to both pH and water activity. The parameter estimation results are presented in Table 4 and the comparison between the logarithm of the measured and predicted growth rates is illustrated in Fig. 3.

Similar as for the case of temperature and pH, the MSE of the gamma model is much higher when attempting to use this model to describe data characterised by both pH and water activity stress than when only describing the separate stresses. Using the gamma-interaction model significantly reduces the value of the MSE to a value even below that of the gamma model on the data that was only stressing with respect to either pH or water activity. It is possible to achieve a lower MSE in this case due to a lower experimental error in the additional data and the use of a highly suitable model structure. The good model fit is also demonstrated by the fact that the MSE is very close to the MSE_{min} , which means that little information is lost by replacing the individual growth rates by the gamma-interaction model. To achieve such a good model fit, a high value of the interaction-parameter β_{pH,a_w} was estimated indicating substantial multiplicative interactions between the effects of pH and water activity. Even though the value of this interaction parameter has a large influence on the overall error between the model and the measurements, the relative uncertainty on its estimated value is high compared to that of the other model parameters. This makes it clear that model predictions have relatively low sensitivity to the value of β_{pH,a_w} and that therefore large

changes in its value may be needed to express the interactions that apply. As the value of β_{pH,a_w} is considerably larger for the combined effect of pH and water activity than for the combined effect of temperature and pH, it can be said that the multiplicative interactions between these first two effects are stronger.

3.4 Modelling the combined effect of temperature and water activity

The model for the combined effect of temperature and water activity was built on the datasets of (i) optimal conditions, (ii) temperature stress, (iii) water activity stress and temperature and water activity stress (iv). A reference for the model parameters of the independent effects was obtained from a parameter estimation of the gamma model on a similar dataset that excluded the combined temperature and water activity stress. The parameter estimation results are listed in Table 5 and the gamma model and gamma-interaction model are compared with the experimental results in Fig. 4.

The values of the estimates of the parameters T_{max} , $a_{w,min}$ and μ_{opt} from the gamma model fitted to the datasets with and without combined temperature and water activity stress are noticeably different. This change in the values of the model parameters points to an unsuitable model structure that is not capable of describing the specific response to the combined temperature and water activity stress. The parameter estimates of the gamma-interaction model on the other hand are very similar to those of the gamma model estimated on the independent effects. Both the ratio between the MSE and MSE_{min} and the visual comparison between the models and the measurements demonstrate that the gamma-interaction model provides a worthwhile improvement over the gamma model to describe the combined effect of temperature and water activity.

Remarkably, for the case of temperature and water activity the parameter β_{T,a_w} was given a negative value during the parameter estimation. As deduced from Eq. (5), negative values of an interaction parameter cause the interaction factor to result in values between 1 and $+\infty$ for gamma factors between 1 and 0, respectively. This can be interpreted as the occurrence of antagonistic interactions between the effects of temperature and water activity on the microbial growth rate of *E. coli*. For the cases of temperature and pH on the one hand and pH and water activity on the other hand, the interaction parameters were positive and therefore represented synergistic interactions.

For negative values of β_{T,a_w} and in case of gamma values equal to 0, the calculation of the total gamma factor $\gamma(T, a_w)$ reduces to:

$$\gamma(T, a_w) = \gamma(T) \cdot \gamma(a_w) \cdot \gamma_i(T, a_w) = 0 \cdot 0 \cdot +\infty \quad (15)$$

As such, the value of $\gamma(T, a_w)$ is indeterminate. However, it can be shown that for combinations of two environmental conditions the overall gamma factor approaches zero for an interaction parameter larger or equal to -1 and approaches a real number for an interaction parameter between -1 and -2. In case of three environmental conditions used in the calculation of the overall gamma factor, β should be larger than or equal to -3. Generally speaking the interaction parameter should be larger than the opposite of the number of environmental conditions included in the calculation of the overall gamma factor. This is illustrated for the cases of two and three environmental conditions in Fig. 5. In this figure, the gamma factor of the combined conditions and interactions is calculated for two (a) or three (b) equal gamma factors of the independent effects and different values of β . This figure illustrates that for some values of β and for gamma factors of the individual effects approaching zero, the resulting gamma factor is asymptotic to $+\infty$. For this reason, the parameter β should be constrained by a lower bound during the parameter estimation to avoid unrealistic mathematical models that

would predict the growth rate approaching $+\infty$ for stressing conditions. However, this also means that the ability of the gamma-interaction model to predict antagonistic interactions is limited and alternatives may be required for cases with very strong antagonistic interactions.

3.5 Modelling the combined effect of temperature, pH and water activity

To develop a model for the combined effect of temperature, pH and water activity on the microbial growth rate of *E. coli* a total of 7 parameter estimations with different combinations of model and dataset were performed. The parameter estimation results are summarised in Table 6. From left to right, the parameter estimations presented in this table are: (i) the gamma model estimated on only optimal conditions and independent stresses, (ii) the gamma model estimated on the full dataset, (iii) the gamma-interaction model including interactions between each combination of two conditions estimated on the dataset that excluded data in which all three conditions were suboptimal, (iv) the same gamma-interaction model estimated on the full dataset, (v) the gamma-interaction model including only interactions between all three conditions estimated on the full dataset, (vi) the gamma-interaction model including interactions between two and three conditions estimated on the full dataset and (vii) the gamma-interaction model including interactions between two and three conditions with an adaptation for strongly antagonistic interactions estimated on the full dataset. The types of interactions that are included in each of the models can also be inferred from the interaction parameters that are estimated.

An attempt to estimate the simple gamma model without any interactions on the full dataset gave poor parameter estimation results. The gamma model was unable to fit the experimental data accurately as seen from the MSE that was about 6 times higher

than the lowest possible value. The change in estimated values of the model parameters such as the pH_{opt} and $a_{w,min}$ also indicates that the model structure is not adequate to describe the measured responses. The parameter estimation of the gamma-interaction model on the datasets a-g gave overall good results, as expected from the finding of the previous sections. As the interaction parameters are uncorrelated, the estimates are highly similar to the ones obtained when separate datasets were used (Tables 3-5). Using the same mathematical model to describe the full dataset resulted in a much higher MSE value. As such, only taking into account interactions between all combinations of two conditions in the model for all three conditions appears to be insufficient to obtain a high accuracy.

Next, a parameter estimation was performed of a gamma-interaction model that only contained an interaction factor for the combined effect of all three conditions. The results of this parameter estimation were comparable with those of the simple gamma model. This can be explained based on the knowledge that the multiplicative interactions between different environmental conditions that were already established varied greatly from antagonistic to strongly synergistic interactions. This leads to the conclusion that a mathematical model for the combined effect of temperature, pH and water activity has to include the interactions between each combination of two conditions and between the combination of all three conditions. As such, the model is formulated as:

$$\gamma(T, pH, a_w) = \gamma(T) \cdot \gamma(pH) \cdot \gamma(a_w) \cdot \gamma_i(T, pH) \cdot \gamma_i(pH, a_w) \cdot \gamma_i(T, a_w) \cdot \gamma_i(T, pH, a_w) \quad (16)$$

The parameter estimation results of this model using the interaction model as elaborated in Eq. (5) are presented in the second to last column of Table 6. This parameter estimation resulted in a good fit and parameter estimates that indicate that

the model extension fits in a modular model building method. However, the interaction parameter β_{T,pH,a_w} was estimated at a value of -14.03. This value indicates strongly antagonistic interactions but, more importantly, such a low value should not be allowed as it results in unrealistic model predictions for values of the gamma factors approaching zero (as demonstrated by Fig. 5). To amend this problem, a simple modification was made to the interaction factor to make it more suitable for the description of antagonistic interactions:

$$\gamma_i(T, pH, a_w) = \left(1 + (1 - \gamma(T)) \cdot (1 - \gamma(pH)) \cdot (1 - \gamma(a_w))\right)^{\beta_{T,pH,a_w}} \quad (17)$$

The parameter estimation results of the gamma-interaction model adapted with this interaction factor are presented in the last column of Table 6. Both based on the parameter estimates and the MSE, this model performed equally well to the previous interaction factor. Moreover, it does not suffer from any constraints on the value of the interaction parameter. As such, this model was found to be the most accurate and suitable model to describe the experimental dataset for the combined effect of temperature, pH and water activity on the growth rate of *E. coli* K12.

The elaborate formulation of $\gamma_i(T, pH, a_w)$ as represented in Eq. (5) can be seen as an alternative to describing this interaction factor with a polynomial that corrects for deviations from the gamma model. However, as established from the current results, the specific systematic structure of the gamma-interaction model contains model parameters that are related to specific stresses as represented by different sections of the dataset. Consequently, this model leads to accurate and uncorrelated estimates of the interaction parameters that benefit reliable model predictions.

3.6 Cross-validation

The previous sections demonstrated that the gamma-interaction model results in a more accurate description of the experimental data than the gamma model. However, it is still unclear from these results whether the significant increase in the model complexity is appropriate to achieve this improvement in the model accuracy. As such, it needs to be demonstrated whether the increased model complexity truly improves the prediction accuracy of the mathematical model and does not result in overfitting. For this reason, a validation study was performed.

An extensive dataset of 68 bioreactor experiments (at 63 distinct conditions) was used to build and evaluate the gamma-interaction model in the previous sections. Because such a large amount of experimental data was already available, a cross-validation was performed. This cross-validation was performed in the following steps: (i) randomised selection of two thirds of the experimental conditions from the full dataset (42 conditions), (ii) parameter estimation of the gamma and gamma-interaction model on the experiments at these conditions, (iii) prediction of the experimental growth rate at the remaining third of the conditions, (iv) calculation of the overall prediction error based on the Mean Prediction Error (MPE):

$$\text{MPE} = \frac{\sum_{k=1}^{N_v} (\ln(\mu_{p,k}(p)) - \ln(\mu_{v,k}))^2}{N_v} \quad (18)$$

In this equation, μ_p is the predicted growth rate, μ_v is the growth rate of the validation experiment determined by primary model parameter estimation and N_v is the number of validation experiments. Growth rates were compared for the validation instead of cell densities as no predicted values of n_0 , q_0 and $n_{\max}$ were required for this comparison. As Akkermans et al. (2016) demonstrated that the error on the logarithm of the growth rate should be minimised to build secondary models in a two-step procedure, the logarithm of the growth rate was used here as well to calculate the MPE.

This procedure (random selection of the dataset, parameter estimation and calculation of the MPE) was repeated 100 times to achieve the spread of both the estimated parameter values and the MPE of the models. Each iteration also contained a multi-start procedure for the parameter estimation with 25 distinct uniformly distributed random sets of initial conditions. The number of initial conditions was selected based on the authors' experience in solving this parameter estimation problem. The randomised selection of the 42 experimental conditions was submitted to the constraints (i) that about 2/3rd of each part of the dataset had to be selected and (ii) that the experiments at a pH of 9.00 and temperature of 44.0°C were always included as these are essential for the estimation of the parameters $T_{\max}$ and $pH_{\max}$. The number of experimental conditions included in the cross-validation is reasonable for the given case study as it lies between the number of experiments included in the commonly used three and four level full factorial designs (27 and 64).

Table 7 presents the cross-validation results. With respect to the parameter estimates this table demonstrates that the variability on the estimated parameters is generally smaller for the gamma-interaction model. Only the variability on the estimated values of pH_{opt} and $pH_{\max}$ is much lower and even negligible. This is due to the fact that these parameters respectively reach their upper and lower bound. The high variability of the parameter estimates of the gamma model signifies high sensitivity of the model predictions to the specific dataset used to build the model. The empirical cumulative distribution of the MPE is also illustrated by Fig. 6. The mean value of the MPE of the gamma model was considerably higher than that of the gamma-interaction model. Moreover, also the spread of the MPE of the gamma model was much larger. This demonstrates that the gamma-interaction model provided more reliable predictions than the gamma model for the current case study. As such, the extensions made by

560 including additional model equations and parameters does not appear to lead to cases
561 of overfitting but rather to a more accurate description of the true response of the
562 microbial growth rate to the environmental temperature, pH and water activity.

563

4 CONCLUSION

Akkermans et al. (2017) revealed the ability of the gamma-interaction model to describe the multiplicative interactions between the effects of temperature and pH better than the competing gamma model, model of Augustin and Carlier (2000) and model of Le Marc et al. (2002). The current research tested the abilities of the gamma-interaction model further, specifically as an extension of the gamma model. The main conclusions are that (i) interactions had to be taken into account for all tested conditions and were successfully added using the gamma-interaction model, (ii) the experimental data needed to estimate the parameters of the gamma-interaction model is inevitable when building any model that accurately describes these combined effects and (iii) the added flexibility is required to achieve a model that provides reliable predictions. These new findings demonstrate that the gamma-interaction model has a large potential to provide food producers and food safety authorities with more accurate and reliable predictions of the microbial growth rate as a function of multiple environmental conditions.

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FIGURE CAPTIONS

Fig. 1. Comparison of the gamma factor of Zwietering et al. (1993) (—) and the CAM (---) with the experimental growth rates (x), 95% confidence bounds) to describe the effect of water activity on the microbial growth rate of *E. coli* K12.

Fig.2. Fit of the gamma model (a) and gamma-interaction model (b) to the data for
the combined effect of temperature and pH on the microbial growth rate and
comparison with the experimental growth rates (●).

Fig.3. Fit of the gamma model (a) and gamma-interaction model (b) to the data for the combined effect of pH and water activity on the microbial growth rate and comparison with the experimental growth rates (●).

Fig.4. Fit of the gamma model (a) and gamma-interaction model (b) to the data for the combined effect of temperature and water activity on the microbial growth rate and comparison with the experimental growth rates (●).

Fig.5. Illustration of the effect of the interaction parameter β on the total gamma factor for the values 2 (—), -1 (—), -2 (—), -3 (—), -4 (—) with two (a) and three (b) influencing factors. This figure illustrates that model outputs are asymptotic to $+\infty$ for some values of β and for gamma factors of the individual effects approaching zero.

Fig.6. Empirical cumulative distributions of the MPE's of the gamma (---) and gamma-interaction model (—) as determined during the cross-validation study.

666

TABLE CAPTIONS

Experiments group	Temperature [°C]	pH [-]	Water activity [-]	Nr.
Optimal (a)	37.0	7.50	0.9949	1 *
Temperature stress (b)	13.0	7.50	0.9949	2 *
	15.4	7.50	0.9949	3
	17.0	7.50	0.9949	4
	21.0	7.50	0.9949	5
	25.0	7.50	0.9949	6
	29.0	7.50	0.9949	7
	33.0	7.50	0.9949	8
	41.0	7.50	0.9949	9
	44.0	7.50	0.9949	10
pH stress (c)	37.0	4.50	0.9949	11
	37.0	4.64	0.9949	12
	37.0	4.80	0.9949	13
	37.0	5.00	0.9949	14
	37.0	5.50	0.9949	15 *
	37.0	6.00	0.9949	16 *
	37.0	6.50	0.9949	17
	37.0	7.00	0.9949	18 *
	37.0	8.00	0.9949	19
	37.0	8.50	0.9949	20
	37.0	9.00	0.9949	21
	37.0	8.75	0.9949	22
Water activity stress (d)	37.0	7.50	0.9502	23
	37.0	7.50	0.9614	24
	37.0	7.50	0.9711	25
	37.0	7.50	0.9779	26
	37.0	7.50	0.9828	27
Temperature and pH stress (e)	17.0	5.00	0.9949	28
	19.0	5.25	0.9949	29
	21.0	5.00	0.9949	30
	21.0	5.50	0.9949	31
	21.0	6.50	0.9949	32
	25.0	5.00	0.9949	33
	25.0	6.00	0.9949	34
	25.0	7.00	0.9949	35
	29.0	4.80	0.9949	36
	29.0	5.50	0.9949	37
	29.0	6.50	0.9949	38
	33.0	7.00	0.9949	39
pH and water activity stress (f)	37.0	5.00	0.9903	40
	37.0	5.25	0.9744	41

	37.0	5.50	0.9670	42
	37.0	5.50	0.9744	43
	37.0	5.50	0.9840	44
	37.0	6.00	0.9667	45
	37.0	6.00	0.9840	46
	37.0	6.50	0.9733	47
Temperature and water activity stress (g)	20.0	7.50	0.9606	48
	20.0	7.50	0.9662	49
	20.0	7.50	0.9754	50
	20.0	7.50	0.9828	51
	25.0	7.50	0.9556	52
	25.0	7.50	0.9609	53
	25.0	7.50	0.9747	54
	30.0	7.50	0.9661	55
	30.0	7.50	0.9750	56
	37.0	7.50	0.9502	57
Temperature. pH and water activity stress (h)	27.0	5.39	0.9815	58
	30.6	5.09	0.9825	59
	30.6	5.09	0.9728	60
	34.4	4.77	0.9929	61
	34.4	5.09	0.9739	62
	34.4	5.09	0.9811	63

667

668 **Table 1.** Overview of the experimental design in eight different categories
669 depending on the stress applied to the microorganism. Experiment performed in
670 duplicate are marked with *.

671

672

	Zwietering et al. (1993)	CAM
$a_{w,min}$ [-]	0.9480 ± 0.0003	0.9370 ± 0.0014
$a_{w,opt}$ [-]	/	0.9970 ± 0.0024
μ_{opt} [h^{-1}]	2.4429 ± 0.0554	2.6165 ± 0.3268
MSE	0.0484	0.0774

673

674 **Table 2.** Parameter estimation results of the gamma factor of Zwietering et al.
675 (1993) and the CAM for the effect of water activity on the microbial growth rate with
676 95% confidence bounds on the parameter estimates.

677

Model	Gamma		Gamma		Gamma-interaction	
Data	a, b, c		a, b, c, e		a, b, c, e	
$T_{\min}$ [°C]	2.3	± 0.3	1.8	± 0.5	1.9	± 0.3
T_{opt} [°C]	40.6	± 0.3	42.0	± 1.2	41.2	± 0.5
$T_{\max}$ [°C]	45.6	± 0.5	44.4	± 0.6	44.9	± 0.4
$\text{pH}_{\min}$ [-]	4.45	± 0.02	4.46	± 0.02	4.37	± 0.05
pH_{opt} [-]	7.14	± 0.16	8.00	± 0.30	7.22	± 0.24
$\text{pH}_{\max}$ [-]	9.02	± 0.01	9.01	± 0.01	9.03	± 0.02
μ_{opt} [h^{-1}]	2.48	± 0.16	2.94	± 0.45	2.67	± 0.16
$\beta_{T,\text{pH}}$	/		/		2.24	± 0.19
MSE	0.0644		0.3079		0.1220	
$\text{MSE}_{\min}$	0.0502		0.0648		0.0648	

Table 3. Parameter estimation results of the gamma and gamma-interaction model on the combined effect of temperature and pH on the microbial growth rate with 95% confidence bounds on the parameter estimates.

Model	Gamma	Gamma	Gamma-interaction
Data	a, c, d	a, c, d, f	a, c, d, f
pH _{min} [-]	4.44 ± 0.02	4.41 ± 0.04	4.41 ± 0.03
pH _{opt} [-]	7.07 ± 0.13	8.90 ± 0.58	6.96 ± 0.14
pH _{max} [-]	9.02 ± 0.01	9.00 ± 0.00	9.03 ± 0.01
a _{w,min} [-]	0.9427 ± 0.0005	0.9446 ± 0.0005	0.9425 ± 0.0005
μ _{opt} [h ⁻¹]	2.21 ± 0.03	2.31 ± .13	2.23 ± 0.03
β _{T,pH}	/	/	9.43 ± 0.69
MSE	0.0460	0.1976	0.0443
MSE _{min}	0.0315	0.0291	0.0291

Table 4. Parameter estimation results of the gamma and gamma-interaction model on the combined effect of pH and water activity on the microbial growth rate with 95% confidence bounds on the parameter estimates.

Model	Gamma		Gamma		Gamma-interaction	
Data	a, b, d		a, b, d, g		a, b, d, g	
$T_{\min}$ [-]	2.3	± 0.4	3.2	± 0.4	2.0	± 0.3
T_{opt} [-]	40.5	± 0.4	39.9	± 0.4	40.8	± 0.3
$T_{\max}$ [-]	45.8	± 0.6	49.5	± 1.4	45.4	± 0.5
$a_{w,\min}$ [-]	0.9426 ± 0.0007		0.9356 ± 0.0011		0.9426 ± 0.0007	
μ_{opt} [h^{-1}]	2.45	± 0.11	2.05	± 0.07	2.47	± 0.11
β_{T,a_w}	/		/		-0.51 ± 0.06	
MSE	0.0750		0.1115		0.0781	
$\text{MSE}_{\min}$	0.0577		0.0528		0.0528	

Table 5. Parameter estimation results of the gamma and gamma-interaction model on the combined effect of temperature and water activity on the microbial growth rate with 95% confidence bounds on the parameter estimates.

Model	Gamma		Gamma		Gamma-interaction		Gamma-interaction		Gamma-interaction		Gamma-interaction		Gamma-interaction ²	
Data	a, b, c, d		a, b, c, d, e, f, g, h		a, b, c, d, e, f, g		a, b, c, d, e, f, g, h		a, b, c, d, e, f, g, h		a, b, c, d, e, f, g, h		a, b, c, d, e, f, g, h	
T_{min} [-]	2.3	± 0.3	2.5	± 0.5	1.9	± 0.3	3.2	± 0.4	2.9	± 0.5	2.1	± 0.3	2.0	± 0.3
T_{opt} [-]	40.6	± 0.3	40.2	± 0.4	41.0	± 0.4	39.7	± 0.4	40.0	± 0.4	40.7	± 0.3	41.0	± 0.4
T_{max} [-]	45.7	± 0.5	46.5	± 0.9	45.0	± 0.4	49.0	± 1.4	47.0	± 1.0	45.5	± 0.5	45.1	± 0.4
pH_{min} [-]	4.45	± 0.02	4.46	± 0.02	4.35	± 0.05	4.37	± 0.05	4.46	± 0.02	4.35	± 0.05	4.34	± 0.05
pH_{opt} [-]	7.09	± 0.15	8.95 ¹	± 0.45	7.07	± 0.21	6.93	± 0.25	8.95 ¹	± 0.49	7.05	± 0.22	7.04	± 0.22
pH_{max} [-]	9.02	± 0.01	9.00	± 0.00	9.05	± 0.11	9.09	± 0.07	9.00	± 0.00	9.07	± 0.05	9.06	± 0.04
$a_{w,min}$ [-]	0.9428	± 0.0006	0.9396	± 0.0012	0.9431	± 0.0007	0.9400	± 0.0013	0.9382	± 0.0015	0.9424	± 0.0008	0.9436	± 0.0008
μ_{opt} [h ⁻¹]	2.50	± 0.08	2.40	± 0.13	2.61	± 0.11	2.07	± 0.05	2.37	± 0.13	2.48	± 0.10	2.58	± 0.12
$\beta_{T,pH}$ [-]	/		/		2.50	± 0.19	3.18	± 0.26	/		2.86	± 0.22	2.84	± 0.23
β_{pH,a_w} [-]	/		/		7.83	± 1.15	4.84	± 0.57	/		8.00	± 1.29	7.51	± 1.40
β_{T,a_w} [-]	/		/		-0.54	± 0.06	-0.30	± 0.11	/		-0.43	± 0.07	-0.50	± 0.07
β_{T,pH,a_w} [-]	/		/		/		/		3.35	± 1.06	-14.03	± 3.08	13.24	± 3.35
MSE	0.0630		0.3221		0.1040		0.1747		0.3123		0.1256		0.1267	
MSE _{min}	0.0473		0.0532		0.0536		0.0532		0.0532		0.0532		0.0532	

¹The estimated value of this parameter has reached the user-defined bound.

²This gamma-interaction model contains an adapted interaction factor for the combined effect of all three conditions taking into account strongly antagonistic interactions. This model is presented in Eq. (17).

Table 6. Parameter estimation results of the gamma and gamma-interaction model on the combined effect of temperature, pH and water

activity on the microbial growth rate with 95% confidence bounds on the parameter estimates.

Model	Gamma		Gamma- interaction	
$T_{\min}$ [-]	2.4	± 0.7	2.1	± 0.3
T_{opt} [-]	40.5	± 0.5	40.9	± 0.3
$T_{\max}$ [-]	46.2	± 1.0	45.3	± 0.4
$\text{pH}_{\min}$ [-]	4.49	± 0.04	4.34	± 0.03
pH_{opt} [-]	8.95	± 0.00	7.11	± 0.09
$\text{pH}_{\max}$ [-]	9.00	± 0.00	9.05	± 0.02
$a_{w,\min}$ [-]	0.9393 ± 0.0022		0.9428 ± 0.0007	
μ_{opt} [h^{-1}]	2.51	± 0.21	2.58	± 0.08
$\beta_{T,\text{pH}}$ [-]	/		2.81	± 0.40
β_{pH,a_w} [-]	/		8.08	± 0.99
β_{T,a_w} [-]	/		-0.42	± 0.14
β_{T,pH,a_w} [-]	/		15.98	± 2.95
MPE	0.0994 ± 0.1039		0.0504 ± 0.0246	

Table 7. Mean parameter estimates and mean model prediction errors obtained during the cross-validation with standard errors.