Including experimental uncertainty on the independent variables when modelling microbial dynamics: The combined effect of pH and acetic acid on the growth rate of *E. coli* K12

Simen Akkermans[a,b,c], Barbara Jaskula-Goiris[d], Filip Logist[a,b,c], Jan F. Van Impe[a,b,c]

[a]BioTeC, Chemical and Biochemical Process Technology and Control, Department of Chemical Engineering, KU Leuven, Ghent, Belgium,

[b]OPTEC, Optimization in Engineering Center-of-Excellence, KU Leuven, Belgium,

[c]CPMF^2, Flemish Cluster Predictive Microbiology in Foods - www.cpmf2.be,

[d]KU Leuven, Faculty of Engineering Technology, Department of Microbial and Molecular Systems (M^{2}S), Cluster for Bioengineering Technology (CBeT), Laboratory of Enzyme, Fermentation and Brewing Technology, Technology Campus, Gebroeders De Smetstraat 1, B-9000 Ghent, Belgium

[simen.akkermans, jan.vanimpe] @kuleuven.be

Correspondence to:

Prof. J. F. Van Impe

Chemical and Biochemical Process Technology and Control (BioTeC)

Department of Chemical Engineering, KU Leuven

Gebroeders de Smetstraat 1, B-9000 Ghent (Belgium)

jan.vanimpe@kuleuven.be

Tel: +32-16-32.14.66

Fax: +32-9-265.86.24

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ABSTRACT

Modelling methods applied in predictive microbiology generally neglect the importance of uncertainty on the measurement of the independent variables. The Ordinary Least Squares (OLS) method that is commonly applied in predictive microbiology is only applicable if the experimental error on the inputs of the model are insignificant. However, this does not apply for many types of experimental measurements of the independent variables. Therefore, a parameter estimation method was adapted in this research for the estimation of the parameters of secondary models, taking into account uncertainty on the measurement of the influencing food characteristics. This parameter estimation method was based on the work of Stortelder (1996) and is referred to as the Weighted Total Least Squares method (WTLS). The method is formulised as an extension of the commonly used OLS method. Consequently the current WTLS method (i) is easily implemented using similar numerical methods, (ii) reduces to an OLS method when the measurement error on the model inputs is negligible and (iii) enables the evaluation of the accuracy of the model parameter estimates based on the same approximations.

Keywords: Predictive microbiology, parameter estimation, total least squares, *Escherichia coli*.

NOMENCLATURE

All symbols in bold and italics represent vectors. All symbols with a single capital letter represent matrices.

$\gamma(\cdot)$	*Relative reduction of the growth rate*
η	*Shape parameter for the pH effect*
κ	*Shape parameter for the pH effect*
μ_{max}	*[h^{-1}] Maximum specific growth rate*
μ_{opt}	*[h^{-1}] Optimum specific growth rate*
ν_m	*Number of experimental measurements*
ν_p	*Number of model parameters*
$\boldsymbol{\pi}$	*Vector of model parameters and experimental errors*
$\boldsymbol{\rho}$	*Vector of weighted residuals and experimental errors*
σ_p^2	*Variance of a model parameter estimate*
σ_{pH}	*Standard deviation on the experimental uncertainty of the pH measurement*
σ_{UAc}	*Standard deviation on the experimental uncertainty of the UAc concentration*
ω	*Shape parameter for the UAc effect*
$\boldsymbol{e}_x$	*Experimental errors on the independent variables*
F	*Fisher information matrix*
J	*Jacobian of the residuals*
MSE	*Mean squared error*
$n(t)$	*[ln(CFU/mL)] Logarithm of the cell density as a function of time*
n_0	*[ln(CFU/mL)] Initial logarithmic cell density*
$\boldsymbol{n}_m(\boldsymbol{t})$	*[ln(CFU/mL)] Cell density measurements*
n_{max}	*[ln(CFU/mL)] Logarithm of the maximum cell density*
$\boldsymbol{n}_p(\boldsymbol{t},\boldsymbol{p})$	*[ln(CFU/mL)] Model predictions of the cell density*
$\boldsymbol{p}$	*Model parameters*
pH_{min}	*Minimum pH for growth*
pH_{opt}	*Optimum pH for growth*
pH_{max}	*Maximum pH for growth*
$q(t)$	*Physiological state of the cell as a function of time*
q_0	*Initial physiological state of the cell*
$\boldsymbol{r}_n$	*Residuals of the cell density measurements*
$\boldsymbol{r}_x$	*Residuals of the independent variables*

SSE	*Sum of squared errors*
UAc	*[ppm] Concentration of undissociated acetic acid*
UAc_{max}	*[ppm] Maximum UAc concentration for growth*
V	*Variance-covariance matrix*
$WMSE$	*Weighted mean squared error*

1 INTRODUCTION

In predictive microbiology, mathematical models are built for describing the responses of microorganisms in food products. The mathematical models used in this field are generally speaking grey box models. Therefore, experimental data is required to calibrate the model by estimating the unknown model parameters. In this parameter estimation, a measure for the distance between the model predictions and the experimental data is minimised by selecting the optimal set of model parameters. The specific method used to estimate the parameters of a mathematical model influences (i) the selection of the most suitable model structure, (ii) the determination of the *optimal* combination of model parameters and (iii) the determination of the accuracy of the model parameters and model predictions. As such, the parameter estimation method has a major influence on the predictions that will be made after constructing a model from a given dataset. Nevertheless, most research in the field of predictive microbiology pays little attention to the selection of appropriate methods for estimating model parameters. The parameter estimation *method* involves (i) a criterion to quantify the distance between the mathematical model and the experimental data and (ii) an algorithm to determine the combination of parameters that optimises this criterion. The optimisation method will mostly influence the time that is required to find a solution and whether the true optimum combination of parameters is found or a combination that is only a local optimum. The optimisation criterion determines how experimental uncertainty and variability is taken into account during modelling. The sources of variation in predictive microbiology were distinguished as follows by Van Impe et al. (2001): (i) the type and quantity of microorganisms in the initial contamination, (ii) the true intrinsic and extrinsic conditions that characterise a food product, (iii) the lack of observations both in the monitoring points and the number of samples, (iv) the random

noise which inevitably corrupts measurements. It is important to consider the effect of these types of variation on the modelling results.

Parameter estimations in predictive microbiology are most often performed following an Ordinary Least Squares (OLS) method. In this method, the optimum combination of parameters is defined as the parameters that minimise the sum of the squared errors between the model predictions and the experimental measurements. The assumptions behind this criterion are that (i) the errors on the independent variables are negligible, (ii) the variance on the errors of all measurements follows a normal distribution, (iii) all measurements are independent observations and (iv) sufficient data is available to obtain a well-spread distribution of the sampling error (Johnson, 1992). If all these assumptions are met, the minimisation of the sum of squared errors is a maximum likelihood estimator, i.e., an objective function that leads to the combination of parameters with the highest likelihood of resulting in the dataset (Walter and Pronzato, 1997). However, when looking at these assumptions in detail it is clear that they are not self-evident when modelling microbial responses in food. For example, data on microbial responses is often relatively sparse as it is labour intensive and expensive to obtain. In general, measurement errors also often not normally distributed.

Moreover, the independent variables that are assumed to be known with high accuracy are also characterised with experimental uncertainty. In the case of measurements such as the environmental temperature, it is indeed a reasonable assumption that the error on the measurement of the influencing factor is negligible compared to the experimental error on the measurement of the microbial response. However, when building mathematical models for microbial responses as a function of, e.g., concentrations of antimicrobials, the experimental error on the measurement of the model inputs will be significant. Uncertainty and/or variability of the input measurements may be overcome

by repeated measurement and calculation of an average when the data has a symmetrical probability distribution. However, this is often impossible or unfeasible because measurements are (i) time consuming, (ii) expensive or (iii) the data was already available and further measurements are no longer possible. For such cases, the parameter estimation procedure should take into account the experimental error on the independent variables and minimise the errors on both the independent and dependent variables taking into account their respective accuracies. This is done by using Weighted Total Least Squares (WTLS). However, such methods are mostly absent from the field of predictive microbiology.

The main exception to the use of basic OLS in predictive microbiology, is the use of Weighted Least Squares (WLS). When using WLS as the optimisation criterion, errors between the model and the measurements are assigned a weight according to estimated accuracy of the measurement. Such a weighted least squares procedure was used, e.g., in Augustin et al. (2000). This publication contained the study of the effect of inoculum size on the lag phase duration of *Listeria monocytogenes*. A weighted least squares fitting criterion was implemented to estimate primary model parameters based on the logarithm of the microbial concentrations. The weights assigned to each measurement were the reciprocal of the variance of the logarithmic concentration. Each variance was determined either from the available replicates or was calculated theoretically based on the various errors occurring during the sampling protocol. Even though such a method takes into account the different errors on the dependent variable, no error on the independent variable is taken into account during the model building.

An exceptional implementation of WTLS in predictive microbiology is found in the research of Baka et al. 2014. In this research, a calibration curve was constructed to relate optical density measurements of cell suspensions to the cell densities in colony

forming units per volume. This calibration curve served as part of the time-to-detection method. As the researchers realised that both the cell density (dependent variable) and the optical density (independent variable) measurements were susceptible to significant experimental uncertainty, they chose to minimise the errors of the model with respect to both the independent and dependent variable. As a way of weighting the different errors, the squared errors were divided by the variance on the measurements. The researchers concluded that the WTLS procedure provided more realistic parameter estimates, compared to the OLS method. The implementation of the WTLS procedure was however limited to a simple linear regression. No other applications of a WTLS procedure were found by the authors in the field of predictive microbiology.

When building mathematical models to describe the effect of environmental conditions on microbial dynamics (growth or survival), a more complex WTLS method is required to take into account the variation on the independent measurements that serve as model inputs. To the author's knowledge, a WTLS has never before been implemented in predictive microbiology research for the purpose of estimating the parameters of a nonlinear model, let alone a model described by algebraic differential equations. As such, the current research deals with the introduction of a WTLS method in the field of predictive microbiology for estimating parameters of nonlinear models. The method is presented in such a way that it is easily compared with the OLS method, and even reduces to OLS under appropriate circumstances. As a case study, the effect of pH in combination with the concentration of undissociated acetic acid (UAc) on the microbial growth rate of *Escherichia coli* K12 was modelled.

This research is aimed at improving the selection of a suitable objective function for parameter estimations in predictive microbiology, while allowing for the same

evaluation methods of the modelling results that are available with conventional methods.

2 MATERIALS AND METHODS

This section discusses the methods used for the experimental case study in which the effect of pH and acetic acid on the microbial growth rate of *Escherichia coli* K12 was modelled. The effect of organic acids on the microbial growth rate is mainly contributed to the concentration of undissociated acid (Beales, 2006). Therefore, the growth rate was modelled as a function of the pH and UAc concentration. The mathematical models used to this end are discussed in Section 2.3.

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 Hearth 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 further 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 the measurement was corrected for temperature variations. pH was controlled by addition of acid (1 N H_2SO_4, 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. Acetic acid (>99.5%, J.T.Baker) was added to the growth medium prior to autoclaving the bioreactor vessel. The pH of the medium was verified externally on a Mettler Toledo SevenCompact pH meter with InLab Routine sensor.

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 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 35 h, depending on the growth rate.

2.1.4 Determination of acetic acid concentrations

Acetic acid concentrations of the autoclaved medium were determined using HPLC-RI. Determination was performed using HPLC Waters system (Waters, Milford, USA) consisting of a Waters 600 controller, a Waters 2414 refractive index detector (HPLC-

RI), a Waters 616 pump, a Waters 717 autosampler and a Waters 600 column oven. Solvent was degassed in line using a Waters AF degasser. Separations were carried out on an Agilent Hi-plex H (7.7 × 300 mm Ø, particle size 8 μm) column (Agilent Technologies, USA) at a temperature of 65°C and a flow rate of 0.6 mL/min. The detector was set at 50°C. The mobile phase was 100% 0.003 M H_2SO_4 (Sigma-Aldrich). Calibration has been performed using acetic acid (>99.5%, J.T.Baker) at concentration levels of 50, 100, 200, 500, 1 000, 2 000 and 5 000 ppm. Data reprocessing was done using Empower 2 software. Prior to HPLC analysis, proteins were removed using Nanosep 3K Omega centrifugal devices (Pall Life Sciences, USA). After centrifugation at 11 000 × g for 5 min, the samples were ready for injection into the HPLC system.

2.1.5 Experimental measurement uncertainty

For the purpose of the WTLS method, the standard deviation of the error on the experimental measurement has to be known to assign a proper weight to the different measurements. The first measurement error that has to be known is the error on the quantification of the microbial population in colony forming units per millilitre. As reported by Van Derlinden et al. (2008), the variance of the error of this measurement was experimentally determined as $3.27 \cdot 10^{-2}$. The square root of this value was used in this research as the standard deviation of the measurement error (σ_n).

In this paper, the effect of the pH and UAc concentration is modelled on the growth rate of *E. coli* K12. The uncertainty on the measurements of both of these independent variables is considered. With respect to the acetic acid concentration, a sample of 500 ppm was measured 20 times to determine the standard deviation of the measured total acetic acid concentration at this mean. The standard deviation of the HPLC measurement was assumed to be linearly proportional to its mean to allow calculation

of the standard deviation over the full range of measurements. Using the well-known Henderson-Hasselbalch equation, the concentration of UAc is calculated at a given pH and total acetic acid concentration. Combining this calculation with a Monte Carlo method enabled the calculation of the standard deviation of the UAc concentration (σ_{UAc}) over a wide range of mean values and pH values. The variation of the pH measurement was taken into account during this simulation as well. The obtained relationship was modelled with a polynomial response surface model for ease of use during further parameter estimations:

$$\sigma_{\mathrm{UAc}} = -21.0 + 11.2 \cdot \mathrm{pH} - 0.295 \cdot \mathrm{UAc} - 1.97 \cdot \mathrm{pH}^2 + 0.108 \cdot \mathrm{pH} \cdot \mathrm{UAc} + 0.115 \cdot \mathrm{pH}^3 - 8.21 \cdot 10^{-3} \cdot \mathrm{pH}^2 \cdot \mathrm{UAc} \quad (1)$$

The values of σ_{UAc} range until about 8 ppm for the experimental conditions used in this study. The variance on the pH measurement was investigated by measuring 6 BHI solutions with different pH values (adapted with 3 M H_2SO_4 addition) spread over the range of tested conditions. The pH of each solution was measured 20 times. The error on the pH measurement appeared to be independent of the mean pH value. As such, the standard deviation on the error of the pH measurement (σ_{pH}) was determined to be $1.65 \cdot 10^{-2}$, independent of the nominal value of the pH.

2.2 Experimental design

A total of 25 bioreactor experiments was performed at distinct combinations of pH and UAc concentration. As this corresponds to the number of experiments present in a 5-level full factorial design, it is a reasonable number of experiments in this type of study. An overview of the selected experimental conditions is presented in Fig. 1. The conditions are represented with respect to the calculated UAc concentration instead of

the total acetic acid concentration as the UAc concentration served as the input for the mathematical model.

2.3 Mathematical models

2.3.1 Primary model

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

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

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

with $\mu_{max}(pH, UAc)$ $[h^{-1}]$ the maximum specific growth rate and $n_{max}(pH, UAc)$ $[\ln(CFU/mL)]$ the maximum cell density for a given pH $[-]$ and UAc concentration (UAc [ppm]). $q(t)$ $[-]$ 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 Secondary models

The combined effect of pH and UAc concentration on the microbial growth rate was described using a gamma model:

$$\mu_{max}(pH, UAc) = \mu_{opt} \cdot \gamma(pH) \cdot \gamma(UAc) \quad (3)$$

with μ_{opt} $[h^{-1}]$ the optimum microbial growth rate, which is only achieved at optimum environmental conditions. $\gamma(pH)$ and $\gamma(UAc)$ represent respectively the relative decrease of the microbial growth rate due to a non-optimal pH and UAc concentration.

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

$$\gamma(\mathrm{pH}) = \left(\frac{(\mathrm{pH}-\mathrm{pH_{min}})^{\eta}\cdot(\mathrm{pH}-\mathrm{pH_{max}})}{(\mathrm{pH}-\mathrm{pH_{min}})^{\eta}\cdot(\mathrm{pH}-\mathrm{pH_{max}})-(\mathrm{pH}-\mathrm{pH_{opt}})^{2}}\right)^{1/\kappa} \quad (4)$$

In this model, the minimum pH, $\mathrm{pH_{min}}[-]$, and the maximum pH, $\mathrm{pH_{max}}[-]$, are the growth boundaries. $\mathrm{pH_{opt}}\,[-]$ 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.

Following the findings of Presser et al. (1997), a mathematical model for the effect of the UAc concentration on the microbial growth rate was formulated as:

$$\gamma(\mathrm{UAc}) = \left(1-\frac{\mathrm{UAc}}{\mathrm{UAc_{max}}}\right)^{\omega} \quad (5)$$

with $\mathrm{UAc_{max}}$ [ppm] the maximum concentration of UAc that permits microbial growth and ω a shape parameter. This mathematical model is generalised with respect to the original model by the addition of ω.

2.4 Computing environment

All parameter estimations are performed using the *lsqnonlin* routine of the Optimisation Toolbox of MATLAB version 7.14 (The Mathworks Inc.). This routine was always combined with a multi-start method with 50 iterations that generated multiple sets of uniformly distributed random initial values of the parameters to be optimised. The lower and upper bounds that were used for these distributions are listed in Table 1.

3 RESULTS AND DISCUSSION

This section starts with the explanation of the commonly used Ordinary Least Squares (OLS) method to estimate the parameters of a mathematical model that describes the evolution of a microbial population. This method only considers the variance on the measurements of the modelled response. Consequently, the Weighted Total Least Squares (WTLS) method is explained for the application of the same type of mathematical models, taking into account the variance on the model inputs as well. This method was applied to an experimental case study in which the microbial growth rate was modelled as a function of measured pH values and UAc concentrations (independent variables). For this case study, the variance on these two measurements was investigated as well. With both methods, a one-step parameter estimation was performed, i.e., the parameters of the primary and secondary model are estimated simultaneously on the dataset of microbial growth curves (dependent variable) at various experimental conditions. The implementation of the one-step parameter estimation method is discussed in more detail in Akkermans et al. (2016).

3.1 The OLS method

The explanation of the OLS method is based on the description found in Walter and Pronzato (1997). The discussion of this method is limited as it is already commonly applied in predictive microbiology and serves as a benchmark for the WTLS method presented in this research. The objective function of a parameter estimation that follows the OLS method is the minimisation of the sum of squared errors (SSE):

$$\text{SSE} = \boldsymbol{r_n}' \cdot \boldsymbol{r_n} \tag{6}$$

with $\boldsymbol{r_n}$ the residual vector. This vector contains the differences between the measured and predicted cell densities at the sampling times in the vector $\boldsymbol{t}$:

$$\boldsymbol{r_n} = \boldsymbol{n}_{\mathrm{m},}(\boldsymbol{t}) - \boldsymbol{n}_{\mathrm{p}}(\boldsymbol{t},\boldsymbol{p}) \quad (7)$$

with $\boldsymbol{n}_{\mathrm{m}}(\boldsymbol{t})$ the vector with the logarithm of the measured cell densities and $\boldsymbol{n}_{\mathrm{p}}(\boldsymbol{t},\boldsymbol{p})$ the vector with the logarithm of the predicted cell densities for a set of parameters $\boldsymbol{p}$. As a measure for the quality of the obtained parameter estimation results, the Mean Squared Error (MSE) is often calculated as:

$$\mathrm{MSE} = \frac{\mathrm{SSE}}{\nu_{\mathrm{m}} - \nu_{\mathrm{p}}} \quad (8)$$

with ν_{m} the number of measurements and ν_{p} the number of model parameters. This MSE provides an unbiased estimate of the variance on the measurement errors. Solving the parameter estimation problem and finding the confidence bounds of the parameter estimates commonly requires the calculation of the Jacobian matrix J of the residuals. Every column of this matrix contains the partial derivative of the elements of the residual vector to one of the model parameters. As the measurements in the residual vector are constants, the Jacobian can be written as:

$$\mathrm{J} = \frac{\partial \boldsymbol{r}}{\partial \boldsymbol{p}} = \begin{bmatrix} \frac{\partial \mathrm{r}_1}{\partial \mathrm{p}_1} & \cdots & \frac{\partial \mathrm{r}_1}{\partial \mathrm{p}_{\nu_\mathrm{p}}} \\ \vdots & \ddots & \vdots \\ \frac{\partial \mathrm{r}_{\nu_\mathrm{m}}}{\partial \mathrm{p}_1} & \cdots & \frac{\partial \mathrm{r}_{\nu_\mathrm{m}}}{\partial \mathrm{p}_{\nu_\mathrm{p}}} \end{bmatrix} \quad (9)$$

Based on J, the information obtained on each model parameter is quantified in the Fisher Information Matrix F, which is calculated as:

$$\mathrm{F} = \frac{1}{\mathrm{MSE}} \cdot \mathrm{J}^{\mathrm{T}} \cdot \mathrm{J} \quad (10)$$

The variance of each parameter estimate is found on the main diagonal of the variance-covariance matrix V. V is approximated by the inverse of F:

$$\mathrm{V} \geq \mathrm{F}^{-1} \quad (11)$$

The 95% confidence interval of every parameter p_{i} is calculated based on the Student's t-distribution (Van Impe et al., 2001):

$$\left[p_i \pm t_{0.975,\nu_m-\nu_p} \cdot \sqrt{\sigma_{p_i}^2}\right] \quad (12)$$

where $\nu_m - \nu_p$ is the number of degrees of freedom. $\sigma_{p_i}^2$ is the variance on the model parameter.

3.2 Adaptation of a WTLS method

The Weighted Total Least Squares (WTLS) method introduced in this research was based on the method elaborated in Stortelder (1996). In their method, the time points that served as model inputs were assumed to have significant variance and the uncertainty on the time points was therefore taken into account. Based on the authors' experience, time points can be determined sufficiently accurate during predictive microbiology studies. On the other hand, measurements of the extrinsic and intrinsic food factors influencing microbial responses are often characterised by significant experimental uncertainty. Measurements with significant experimental uncertainty are, e.g., pH, concentrations of antimicrobials or atmosphere composition. As such, the WTLS method explained below is aimed at taking into account errors on such measurements as well.

The idea of the WTLS method is that apart from minimising the errors between the measurements and the model of the dependent variable, which are listed in $\boldsymbol{r}$, also the errors between the model and one or more dependent variables are considered. Assume that a set of independent variables $\boldsymbol{x}$ is taken into account and that the errors on these measurement are $\boldsymbol{e}_x$. The independent variable is measured once for every experiment but the error of this measurement is obviously unknown. Consequently, in this WTLS method, all errors on the measurements of the independent variables are estimated as model parameters. Consequently, a new parameter vector $\boldsymbol{\pi}$ is defined as:

$$\boldsymbol{\pi} = \begin{bmatrix} \boldsymbol{p} \\ \boldsymbol{e}_{\mathrm{x}} \end{bmatrix} \tag{13}$$

As such, the errors on the measurements of the independent variables are estimated, just like the model parameters. However, any deviation of the values of $\boldsymbol{e}_{\mathrm{x}}$ from zero has to be penalised as well, same as any deviation between the model predictions and measurements of the dependent variable. For this purpose, $\boldsymbol{e}_{\mathrm{x}}$ is implemented in a new residual vector $\boldsymbol{\rho}$. In $\boldsymbol{\rho}$, the errors are weighted inversely proportional to the standard deviations of the measurement errors σ_{n} and σ_{x}:

$$\boldsymbol{\rho} = \begin{bmatrix} \frac{1}{\sigma_{\mathrm{n}}} \boldsymbol{r}_{\mathrm{n}} \\ \frac{1}{\sigma_{\mathrm{x}}} \boldsymbol{e}_{\mathrm{x}} \end{bmatrix} \tag{14}$$

The residual vector for the model outputs $\boldsymbol{r}_{\mathrm{n}}$ is calculated at the corrected model inputs $\boldsymbol{x} + \boldsymbol{e}_{\mathrm{x}}$ (see Figure 2). The weighting is needed to take into account the difference in accuracy between different types of experimental measuring techniques. The objective function of the WTLS method is consequently written as follows:

$$\min_{\boldsymbol{\pi}} \boldsymbol{\rho}^{\mathrm{T}} \cdot \boldsymbol{\rho} \tag{15}$$

Using the newly define parameter vector $\boldsymbol{\pi}$ and residual vector $\boldsymbol{\rho}$, the calculation of the confidence bounds is performed similarly to the OLS method. The Jacobian matrix is calculated as:

$$\mathrm{J} = \frac{\partial \boldsymbol{\rho}}{\partial \boldsymbol{\pi}} \tag{16}$$

Due to the inclusion of the standard deviations in the residual vector $\boldsymbol{\rho}$, the calculation of F is simplified. It can be demonstrated that the division by the MSE, which is a measure for the variance on the measurement error, needs to be omitted as it is already included in J (see Appendix A). The calculation of F becomes:

$$\mathrm{F} = \mathrm{J}^{T} \cdot \mathrm{J} \tag{17}$$

The remainder of the calculation of the variance-covariance matrix and the confidence bounds of the model parameters and the measurement errors is completely analogous to the explanation in Section 3.1. The advantage of using the WTLS method as defined in this section is that, through its analogy to the OLS method, it can easily be solved by already available methods such as the *lsqnonlin* function of MATLAB.

The evaluation of the quality of the fit for the OLS method is commonly done using the value of the MSE, as this value takes into account both the overall error between the model and the measurements and the number of parameters with respect to the number of measurements (degrees of freedom). The MSE is extended to the WTLS method as well. However, in this case, the squared error is not only calculated between the model output and the experimental measurements but also between the estimated model inputs $\boldsymbol{x}_\mathrm{m} + \boldsymbol{e}_\mathrm{x}$ and the model inputs that correspond to the measurement of the dependent variable $\boldsymbol{x}'$. This error is called the residual of the independent variable $\boldsymbol{r}_\mathrm{x}$, analogous to $\boldsymbol{r}_\mathrm{n}$. These calculation of both residuals is illustrated in Fig. 2. For the OLS method, the same calculation of the residuals as presented in this figure holds but the estimated error on the model input ($\boldsymbol{e}_\mathrm{x}$) is always equal to zero. The Weighted Mean Squared Errors (WMSE) of measured cell densities ($\mathrm{WMSE_n}$) and independent variables ($\mathrm{WMSE_x}$) are calculated as follows:

$$\mathrm{WMSE_n} = \frac{1}{\sigma_\mathrm{n}^2} \cdot \frac{\boldsymbol{r}_\mathrm{n}^\mathrm{T} \cdot \boldsymbol{r}_\mathrm{n}}{\nu_\mathrm{m} - \nu_\mathrm{p}};\ \mathrm{WMSE_x} = \frac{1}{\sigma_\mathrm{x}^2} \cdot \frac{\boldsymbol{r}_\mathrm{x}^\mathrm{T} \cdot \boldsymbol{r}_\mathrm{x}}{\nu_\mathrm{m} - \nu_\mathrm{p}}$$

The WMSE expresses the error between the model and the measurements relative to the experimentally determined uncertainty on the measurement. Compared to the OLS method, both ν_m and ν_p increase with the number of measurements of the independent variable. As such, the degrees of freedom of the parameter estimation problem remains unchanged. The WMSEs can be calculated for the OLS method as well. In some cases,

data points may have a higher measured value than any model output. This means that there is no model prediction that corresponds to the measurement of the independent variable. As such, it is impossible to calculate the distance between the data point and the model for the residual of the independent variable. In such cases, the distance to the optimum value of the model can be taken.

With respect to the case study used in this research to demonstrate the use of the WTLS method, $\boldsymbol{\pi}$ and $\boldsymbol{\rho}$ are defined as:

$$\pi = \begin{bmatrix} \boldsymbol{p} \\ \boldsymbol{e}_{\mathrm{pH}} \\ \boldsymbol{e}_{\mathrm{UAc}} \end{bmatrix}; \rho = \begin{bmatrix} \frac{1}{\sigma_{\mathrm{n}}} \boldsymbol{r_n} \\ \frac{1}{\sigma_{\mathrm{pH}}} \boldsymbol{e}_{\mathrm{pH}} \\ \frac{1}{\sigma_{\mathrm{UAc}}} \boldsymbol{e}_{\mathrm{UAc}} \end{bmatrix} \tag{18}$$

with $\boldsymbol{e}_{\mathrm{pH}}$ and $\boldsymbol{e}_{\mathrm{UAc}}$ the estimated errors on the measurements of the pH and UAc concentration and σ_{pH} and σ_{UAc} the standard deviations of these measurement errors.

3.3 Modelling the effect of acetic acid

To build a mathematical model for the effect of UAc on the growth rate of *E. coli*, all experiments at pH 6.00 were selected from the dataset described in Section 2.2. As such, five experiments at different acetic acid concentrations were selected at a constant pH. The parameters of the model in Eq. (5) were estimated on this dataset according to both the OLS and WTLS method. With each method, two alternatives for this model were tested. In the first, the parameter ω was fixed at a value of 1, to obtain a linear relationship between the concentration of UAc and the microbial growth rate. In the second model, the value of ω was estimated. The parameter estimation results are presented in Table 2. Parameter estimates are presented with 95% confidence bounds as determined according to the methods explained in Sections 3.1 and 3.2. During the parameter estimation, values of n_0 and q_0 were estimated for every growth curve. Since

these have little effect on the determination of the secondary model parameters, they were omitted from the results shown here. For both parameter estimation methods the WMSEs of the logarithmic cell densities (model output, $WMSE_n$) and the UAc concentration (model input, $WMSE_{UAc}$) are calculated. When calculating the $WMSE_n$ for the OLS method, this comes down to dividing the regular MSE by the experimentally determined variance on the measurement error. The $WMSE_{UAc}$ for the OLS method is calculated in exactly the same manner as for the WTLS method, but the estimated measurement errors on the UAc concentrations are equal to zero. The outputs of the four models are compared with each other and with the experimental data in Fig. 3. The comparison is based on the logarithm of the growth rate predicted by the model and estimated from each growth curve using the primary model of Eq. (2).

Fig. 3 illustrates the difference between (i) the models with fixed and estimated value ω and (ii) the OLS and WTLS method. The predictions of the two models with a value of ω equal to 1 diverge for high UAc concentrations. From this figure, it appears as if the OLS method approximates the data points of the microbial growth rate more closely. However, by assigning an estimated measurement error to the different UAc concentrations, the WTLS method shifts data points in the horizontal direction. Changing the model inputs for the UAc concentrations by means of the addition of an experimental error is penalised in the objective function as explained in Section 3.2. Fig. 4 illustrates the fit of the two models on the measured cell densities. This figure clarifies that both methods make a very good and almost the same approximation of the growth as a function of time for various UAc concentrations. As seen from the $WMSE_{UAc}$ and $WMSE_n$ in Table 2, the WTLS method is able to estimate experimental errors on the UAc concentrations in such a way that the error between the measured and predicted cell densities is further decreased. Naturally, also the error between the

model and the measurements of the UAc concentration is decreased significantly in the WTLS method. There is a small difference in the estimated values of the model parameters. However, it is not possible to say that these are significantly different considering that the 95% confidence bounds of both methods overlap. The uncertainty on the parameter estimates is higher with the WTLS method, as this method takes into account uncertainty on the model inputs as well. It is expected that this higher uncertainty is more realistic than the estimated uncertainty of the OLS method that assumes the measurements of the UAc concentrations to be exact. After all, the estimated uncertainty on the model parameters is an approximation under the assumption that no errors exist on the model input, an assumption that does not hold in this case.

The difference between the models that result from the two methods when estimating the value of ω as well is less clear in Fig. 3. Based on the WMSEs in Table 2, it is clear that the models from both methods approximate the dataset very closely. The parameter estimation results in the same table show that, both for the OLS and WTLS method, the introduction of ω as a model parameter leads to very high uncertainty on the parameter estimate. This high uncertainty is found despite the good approximation of the experimental measurements. As such, this high uncertainty on the estimated values of the model parameters is due to a case of over fitting. The model is very closely related to the experimental data but the model parameters can no longer be estimated accurately as the data provides too little information on their exact value. The reason that there is so little difference between the estimated models from both methods is that the WTLS method reduces to an OLS method if the errors on the measurements of the independent variables are considered negligible. In any case, both methods demonstrate that the model with a value of ω to be estimated is too complex for the given dataset.

For the model with ω fixed to 1, a leave-one-out cross-validation was performed. In this validation, the model parameters were estimated with both methods on all but one of the experiments. Then, the predicted growth rate was compared with the remaining experiment. The mean squared prediction error was found to be 0.056 for the OLS method and 0.079 for the WTLS method. However, when correcting the measured concentration of undissociated acetic acid for that which was determined in the full WTLS method, the errors changed to 0.037 and 0.030 respectively. Consequently, whether or not the WTLS method is more accurate than the OLS method is really dependent on the errors that exist on the measurement of the independent variables.

Based on the findings of this section, the model of Eq. (5) for the effect of the UAc concentration on the microbial growth rate was simplified by fixing ω to a value of 1. Both the graphical representations (Fig. 3 and 4) and the quantitative results (Table 3) demonstrated the fitting quality of this model. This simplified model was used in the next part of this research paper to model the combined effect of pH and UAc concentration.

3.4 Modelling the effect of pH and acetic acid

In this section, three different models for the combined effect of pH and UAc concentration were tested on a dataset containing 25 distinct experimental conditions. These three models only differ in their complexity with respect to the description of the effect of pH on the relative reduction of the microbial growth rate. In the most complex model, the shape parameters κ and η are both estimated. A first simplification is made by fixing the parameter η to a value of 1. In this model, the equation is symmetrical for both the suboptimal and superoptimal effect of pH on the growth rate. A further simplification is made by fixing both κ and η to 1. In this case, the pH model is exactly

the same as the model published by Rosso et al. (1995). The aim of this modelling study is to evaluate the applicability of the WTLS method to more complex model building exercises that contain multiple measurements of dependent variables, each with a characterised experimental uncertainty.

The results of the parameter estimations with both the OLS and WTLS method are presented in Table 3. Looking first at the three different models, it is clear that some identification problems arise when estimating the value of both κ and η. Irrespective of the parameter estimation method used, the confidence bounds on the estimated values of all model parameters related to the pH effect are excessive. The reason behind this high uncertainty lies in the scope of the experimental conditions that were used during the parameter estimation. No information is available on the microbial response for pH values above 7.00, i.e., above the approximate optimum pH. As such, the data does not allow the determination of the asymmetry between the suboptimal and superoptimal effect of pH, which is characterised by the value of η. Consequently, it is not possible to accurately determine the optimum combination of model parameters. The origin of this problem is endorsed by the parameter estimation results of the model in which η was fixed to a value of 1. For this model equation, all model parameters were estimated with high accuracy for both methods. These findings demonstrate that the WTLS method, as discussed in Section 3.2, allows the study of the accuracy of the model parameter estimates in the same way as the basic OLS method. The same conclusions should be drawn with respect to the suitability of the model structure (complexity) when using the WTLS method instead of the OLS method. The very large $WMSE_{pH}$ values are a consequence of the plateau shape of the pH model close to the optimal conditions. This shape leads to large differences between the experimentally measured pH and the pH of the model at the same growth rate. As such, it is clear that the WMSE of the

independent variables is only useful for comparison between competing models, but not as a measure for the quality of fit on its own.

For both methods, the cell density measurements were approximated slightly better by the model with an estimated value of κ than by the model with a fixed parameter, as seen from the value of the $WMSE_n$. The model with $\kappa = 1$ has a much larger error between the UAc concentrations of the measurements and those of the model but a smaller error between the pH measurements and model. It is clear that the OLS method does not take this error into account during the parameter estimation procedure. However, even though the WTLS method has additional possibilities to affect the error between the model and the measurements of the pH and UAc concentrations, the minimisation of this error is not part of the objective function. Adding an estimated error to the measurement of one of the independent variables may improve the overall value of the objective function, but only if the decrease of the error on the measured cell density is larger than the increase of the estimated error on the measured pH or UAc concentration (considering their respective weights). It is clear that the discrepancy between the model and the input variables is much smaller for the WTLS method than for the OLS method. But the existence of slightly higher differences between the model and input variables in the WTLS method should not be given too much importance because the fact that these errors remain means that the model output already closely approximates the measurements of the cell density. The estimated errors are up to about 20 ppm for the UAc measurements and up to 0.02 for the pH measurement. These estimated experimental errors are plausible taking into account the experimentally determined measurement uncertainty. These results show that the WTLS method allows for comparison of the quality of fit of different models through the WMSE, similarly as with the MSE when working with the OLS method. In this case study, it is the WMSE

of the cell densities that is most important for the comparison of the model fits. Generally speaking, the WMSE on the output of the model is of most relevance to evaluate the model fit.

4 CONCLUSION

In this work, a WTLS method was introduced in predictive microbiology and adapted for the specific application of estimating the parameters of secondary models. The WTLS method that was obtained is formulated as an extension of the more commonly used OLS method. Consequently, the method can also reduce automatically to an OLS method when there is no need to take errors on the independent measurements into account. This was seen in the results when the experimental dataset was very closely approximated by the mathematical model. Also the calculation of the confidence bounds on the model parameter estimates is an extension of the commonly used OLS method. The main difference here is that the uncertainty on the measurements of the model inputs is neglected in the OLS method but taken into account in the WTLS method. Even though the number of measurements increases in the WTLS method, the number of model parameters increases with the same amount. As such, the predicted uncertainty on the model parameter estimates will generally be larger for the WTLS method but may better reflect reality.

The evaluation of the fit of the mathematical model to the experimental dataset was done using the WMSE of the different dependent and independent variables. However, it was concluded that it is mainly important to consider the WMSE of the output of the model, which was the logarithm of the cell density is this research. The WMSE of this model output is analogous to the MSE used in the more basic OLS method. The WMSEs of the independent variables give an indication of the difference between the model and the experimental measurements as well, but this is not taken into account during the parameter estimation.

With respect to the implementation of the parameter estimation problem, the main difficulty lies in solving the one-step parameter estimation but there was no real

difference as opposed to solving a one-step parameter estimation according to an OLS method. By formulating the WTLS method analogous to the OLS method, it was implemented easily in MATLAB using the *lsqnonlin* function. Even though a multi-start procedure was applied, in most cases the same optimum solution was found.

This research demonstrated the possibility of taking into account experimental uncertainty on the measurement of independent variables to estimate the parameters of secondary models in predictive microbiology. In all cases where the measurement uncertainty is easily characterised, the commonly applied OLS method is easily extended to a more realistic WTLS method. Moreover, such a WTLS method is indeed recommended to take errors on the measurements of independent variables into account when they are significant compared to the errors on the dependent variables. As such, the WTLS method can contribute to more accurate models for predicting microbial dynamics as a function of intrinsic and extrinsic characteristics of food products.

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APPENDIX A

This section demonstrates that the Fisher information matrix for the parameter estimates of the WTLS method is calculated as follows:

$$F = J^T \cdot J \tag{19}$$

Substitution of the Jacobian as defined in Eq. (16) results in the expression:

$$F = \frac{\partial \boldsymbol{\rho}}{\partial \boldsymbol{\pi}}^T \cdot \frac{\partial \boldsymbol{\rho}}{\partial \boldsymbol{\pi}} \tag{20}$$

Assuming that the distribution of the measurement error is equal for all measurements, the residual vector $\boldsymbol{\rho}$ can be simplified to $\boldsymbol{r}/\sigma$ with $\boldsymbol{r}$ the residual vector of all considered measurements and σ the standard deviation of the errors. This simplification is substituted into Eq. (20).

$$F = \left(\frac{1}{\sigma}\frac{\partial \boldsymbol{r}}{\partial \boldsymbol{\pi}}\right)^T \cdot \frac{1}{\sigma}\frac{\partial \boldsymbol{r}}{\partial \boldsymbol{\pi}} = \frac{1}{\sigma^2}\frac{\partial \boldsymbol{r}}{\partial \boldsymbol{\pi}}^T \cdot \frac{\partial \boldsymbol{r}}{\partial \boldsymbol{\pi}} \tag{21}$$

This above expression for the Fisher information is equivalent to Eq. (10), for the OLS method because (i) $\partial \boldsymbol{r}/\partial \boldsymbol{\pi}$ corresponds to the Jacobian matrix as defined in a standard OLS parameter estimation and (ii) σ^2 is the variance on the measurement error that is approximated by the MSE. As such, Eq. (19) can be used to calculate the Fisher information matrix for the WTLS method.

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FIGURES

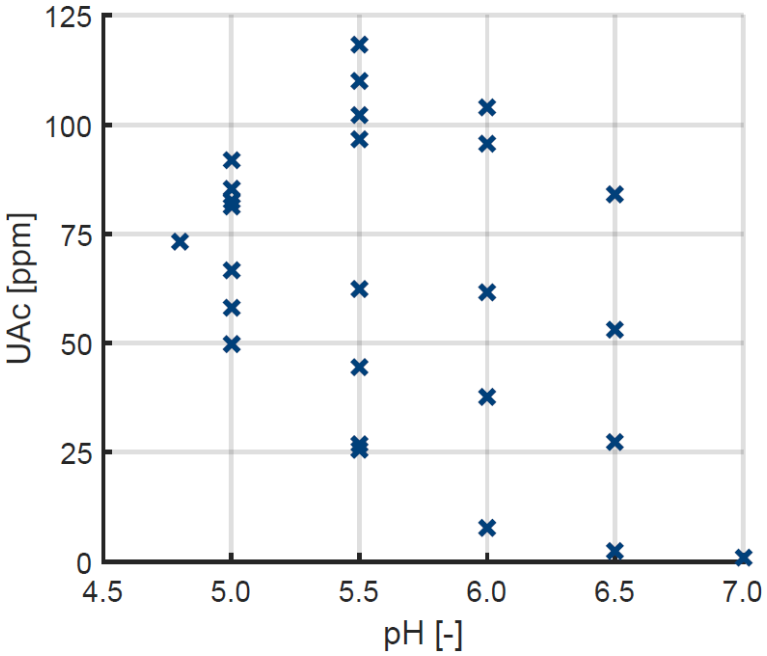

Fig. 1. Experimental dataset as a function of the pH and concentration of undissociated acetic acid (UAc).

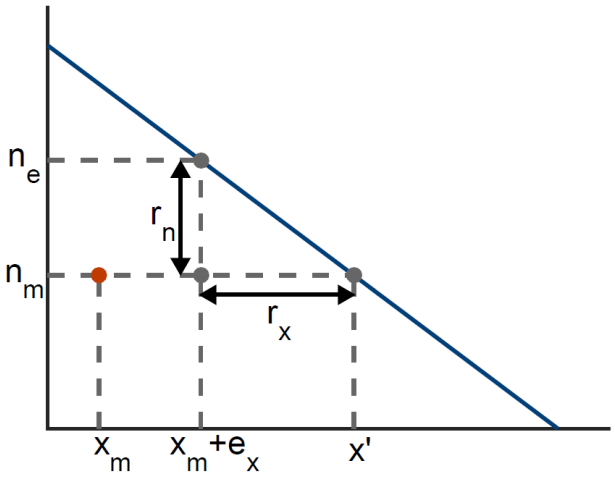

Fig. 2. Illustration of the calculation of the residual on the independent and dependent variable ($\mathbf{r_x}$ and $\mathbf{r_n}$) according to the WTLS method. $\mathbf{x_m}$ and $\mathbf{n_m}$ are the measurements of the independent and dependent variable. $\mathbf{x_m} + \mathbf{e_x}$ is the measurement of the independent variable that is corrected for the estimated measurement error and $\mathbf{n_e}$ is the model output corresponding to this input. $\mathbf{x}'$ is the value of the independent variable that results in the measured dependent variable according to the model equation.

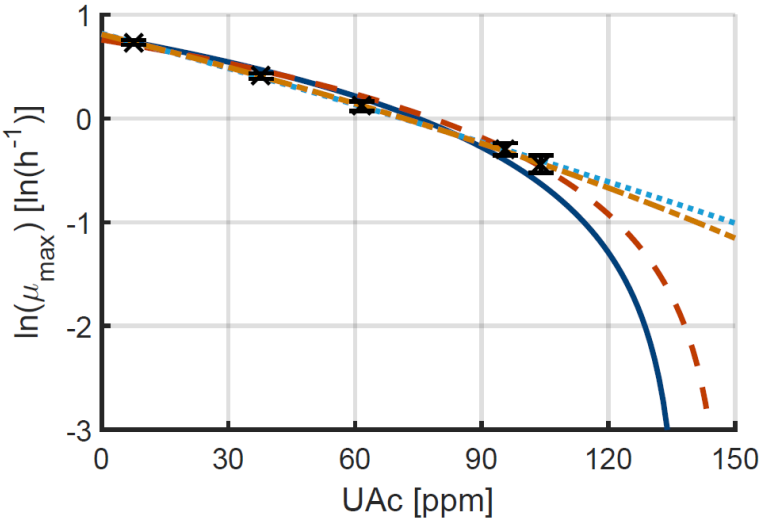

Fig. 3. Comparison of two different models for the effect of undissociated acetic acid on the microbial growth rate estimated with both the OLS and WTLS method and comparison with the experimental growth rates (**X**). The curves are the (i) model with $\boldsymbol{\omega} = \mathbf{1}$ and OLS method (---), (ii) model with $\boldsymbol{\omega}$ estimated and OLS method (···), (iii) model with $\boldsymbol{\omega} = \mathbf{1}$ and WTLS method (—) and (iv) model with $\boldsymbol{\omega}$ estimated and WTLS method (⋯).

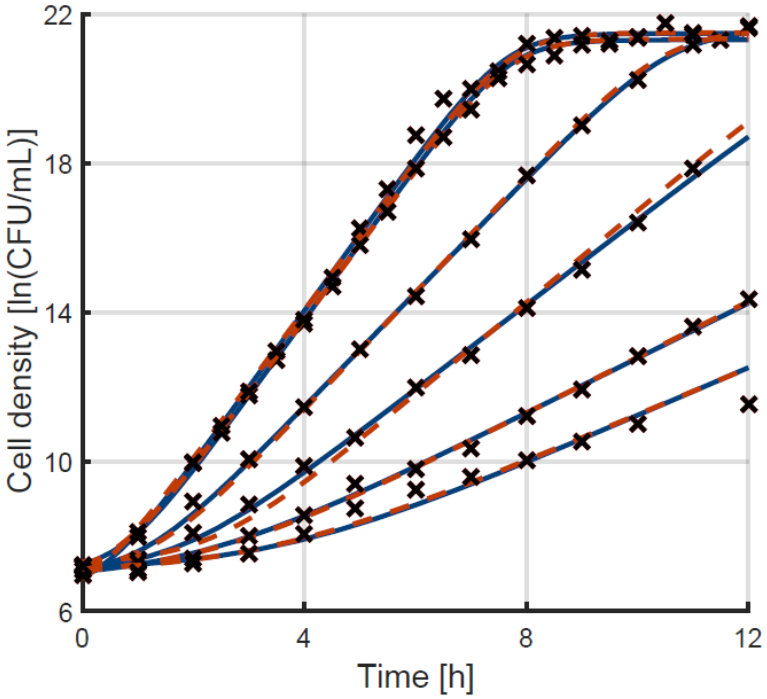

Fig. 4. Comparison of the model with $\boldsymbol{\kappa} = \mathbf{1}$ according to the OLS (---) and WTLS (—) method with the experimentally measured cell densities (**X**).

TABLES

Parameter	Lower bound	Upper bound
pH_{min} [-]	4.00	5.00
pH_{opt} [-]	7.00	8.00
κ [-]	1.00	10.00
η [-]	1.00	10.00
UAc_{max} [ppm]	100.0	1000.0
ω [-]	0.00	50.00
μ_{opt} [h^{-1}]	0.500	3.000

Table 1. A list of the lower and upper bounds that were used during the parameter estimation for the different model parameters. These bounds also defined the uniform distributions that were used for the multi-start method.

Method	OLS		OLS		WTLS		WTLS	
UAc_{max} [ppm]	148.7	± 3.7	327.5	± 254.6	138.9	± 12.0	398.9	± 877.6
ω [-]	1[1]		3.23	± 3.06	1 [1]		4.05	± 10.1
μ_{opt} [h^{-1}]	2.102	± 0.057	2.227	± 0.083	2.163	± 0.066	2.260	± 0.098
$WMSE_n$	1.958		1.630		1.607		1.592	
$WMSE_{UAc}$	3.409		0.002		0.936		0.128	

Table 2. Parameter estimation results for the model of the effect of UAc concentration on the microbial growth rate as estimated with the OLS and WTLS method.

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The content is identical to the published paper, but without the final typesetting by the publisher.

Method	OLS		OLS		OLS		WTLS		WTLS		WTLS	
pH_{min} [-]	4.06	± 0.20	4.49	± 0.53	4.13	± 8.83	3.85	± 0.28	4.49	± 0.51	4.26	± 4.06
pH_{opt} [-]	6.50	± 0.14	6.61	± 0.28	8.90	± 64.00	6.47	± 0.12	6.50	± 0.20	8.89	± 28.11
κ [-]	1[1]		1.81	± 1.62	4.86	± 32.97	1[1]		2.01	± 1.72	8.04	± 36.00
η [-]	1[1]		1[1]		5.13	± 37.35	1[1]		1[1]		5.48	± 17.01
UAc_{max} [ppm]	170.7	± 5.1	170.3	± 5.05	167.6	± 4.7	150.3	± 6.9	150.0	± 6.8	149.5	± 5.5
μ_{opt} [h^{-1}]	2.140	± 0.047	2.119	± 0.052	2.083	± 0.124	2.232	± 0.040	2.207	± 0.047	2.177	± 0.060
$WMSE_n$	3.119		3.043		2.933		1.288		1.269		1.253	
$WMSE_{UAc}$	44.225		26.005		33.612		12.752		5.755		1.087	
$WMSE_{pH}$	64.616		71.111		640.908		10.686		12.118		118.346	

[1]For these parameter estimations, the value of κ or η was fixed to 1. As such the model for the effect of pH on the microbial growth rate is simplified as explained in Akkermans et al. (2017). The value of ω of the UAc model is fixed to 1 for all parameter estimations.

Table 3. Parameter estimation results for the model of the combined effect of pH and UAc concentration on the microbial growth rate resulting from both the OLS and WTLS method. Three models with a different level of complexity were considered by fixing the values of κ and η to 1.