



## Food Microbiology

# Predictive modeling of *Pseudomonas fluorescens* growth under different temperature and pH values



Letícia Dias dos Anjos Gonçalves<sup>a,\*</sup>, Roberta Hilsdorf Piccoli<sup>b</sup>,  
Alexandre de Paula Peres<sup>b</sup>, André Vital Saúde<sup>c</sup>

<sup>a</sup> Universidade Federal do Triângulo Mineiro, Departamento de Engenharia de Alimentos, Uberaba, MG, Brazil

<sup>b</sup> Universidade Federal de Lavras, Departamento de Engenharia de Alimentos, Lavras, MG, Brazil

<sup>c</sup> Universidade Federal de Lavras, Departamento de Ciência da Computação, Lavras, MG, Brazil

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## ABSTRACT

Meat is one of the most perishable foods owing to its nutrient availability, high water activity, and pH around 5.6. These properties are highly conducive for microbial growth. Fresh meat, when exposed to oxygen, is subjected to the action of aerobic psychrotrophic, proteolytic, and lipolytic spoilage microorganisms, such as *Pseudomonas* spp. The spoilage results in the appearance of slime and off-flavor in food. In order to predict the growth of *Pseudomonas fluorescens* in fresh meat at different pH values, stored under refrigeration, and temperature abuse, microbial mathematical modeling was applied. The primary Baranyi and Roberts and the modified Gompertz models were fitted to the experimental data to obtain the growth parameters. The Ratkowsky extended model was used to determine the effect of pH and temperature on the growth parameter  $\mu_{max}$ . The program DMFit 3.0 was used for model adjustment and fitting. The experimental data showed good fit for both the models tested, and the primary and secondary models based on the Baranyi and Roberts models showed better validation. Thus, these models can be applied to predict the growth of *P. fluorescens* under the conditions tested.

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## Introduction

Fresh meat is a highly perishable product owing to its high nutritious content. Many interrelated factors influence the shelf life and freshness of meat such as storage temperature, atmospheric oxygen (O<sub>2</sub>), endogenous enzymes, moisture, light and most importantly, micro-organisms.<sup>1</sup> Therefore,

meat is a complex niche with particular physicochemical characteristics that allow colonization and development of a wide variety of organisms,<sup>2</sup> making it highly susceptible to be contaminated by spoilage bacteria.<sup>3</sup> Microbial spoilage can lead to food disposal and consequent economic losses to the industry and a decrease in consumer trust.<sup>4</sup>

Various conservation methods are available to increase the shelf life of fresh meat including refrigeration.<sup>5</sup> The use of cold

\* Corresponding author.

E-mail: [leticiauftm@gmail.com](mailto:leticiauftm@gmail.com) (L.D. Gonçalves).

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temperatures, such as refrigeration and freezing, is considered the most effective method of retarding or inhibiting microbial growth in meat products during transportation or storage for maintaining product quality and extending shelf life.<sup>6</sup> The use of refrigeration to preserve food is based on the reduced rate of metabolism in microorganisms at low temperatures.<sup>7</sup> The rate of enzyme-catalyzed reactions is temperature dependent, and considerably slows down at low temperatures. Therefore, it is extremely important to control and maintain the refrigeration temperature within the acceptable limits to ensure the security and integrity, and to extend the shelf life of meat products.<sup>8</sup>

However, even in cold stored meat, the organoleptic spoilage by microbial consumption of meat nutrients, such as sugars and free amino acids, and the release of undesired volatile metabolites cannot be eliminated completely. These activities may be performed at low temperatures by psychrotrophic bacteria compromising preserving effect of low temperature.<sup>5</sup> These microorganism can thrive well at low temperatures via modifying their cytoplasmic membrane and increasing the unsaturated fatty acids levels, which keep the membrane in semifluid state, thereby facilitating the transport of nutrients and enzymes.<sup>9</sup> Their ability to grow at low temperatures is one of the challenges to the meat industry while considering the meat quality and public health.<sup>10</sup>

*Pseudomonas* spp. stand out among the psychotropic microorganisms, particularly, those involved in the spoilage of meat stored at low temperatures.<sup>11</sup> They are Gram-negative, aerobic, proteolytic and lipolytic microorganisms mostly associated with the deterioration of fresh meat. The most commonly found pseudomonas in meat is *Pseudomonas fluorescens*, which is characterized by producing a soluble pigment called pyoverdine.

Predictive microbiology can be used to assess the quality of food susceptible to deterioration by these spoiling microorganisms considering the fact that the behavior of the microbial population and its effect on environment can be predicted, as its status is known at any given time.<sup>12</sup> Thus, predictive models can be used to predict product quality and microbiological safety considering the product characteristics and processing and storage conditions.<sup>13</sup>

This study aimed to construct and validate the mathematical models for predicting *P. fluorescens* growth at different temperatures and pH conditions.

## Material and methods

### Inoculum standardization and maintenance

Freeze-dried *P. fluorescens* strain ATCC 13525 was stored at  $-18^{\circ}\text{C}$  in a freezing medium containing 15 mL glycerol, 0.5 g bacteriological peptone, 0.3 g yeast extract, 0.5 g NaCl, and 100 mL distilled water. Prior to use, the strain was activated in brain heart infusion (BHI) broth (HiMedia, Mumbai, India) and incubated at  $28^{\circ}\text{C}$  for 24 h to obtain the number of cells necessary for standardization.

### Effect of storage temperature and pH of the medium on the growth of *P. fluorescens* in meat broth

Aliquots of the standardized inoculum were transferred to 100 mL meat broth (10 g meat extract, 10 g meat peptone, 5 g tryptone, and 5 g glucose in 1 L water) at a final concentration of  $10^4$  CFU  $\text{mL}^{-1}$ , and incubated at  $4^{\circ}\text{C}$ ,  $7^{\circ}\text{C}$ , and  $12^{\circ}\text{C}$ .

The pH of the medium was adjusted to 5.5, 6.0, and 6.3 with 2 M NaOH or 2 M HCl using a digital pH meter (Digimed DM20, Campo Grande, Brazil).

The growth of *P. fluorescens* at each pH and temperature condition ( $4^{\circ}\text{C}$  and pH 5.5;  $4^{\circ}\text{C}$  and pH 6.0;  $4^{\circ}\text{C}$  and pH 6.3;  $7^{\circ}\text{C}$  and pH 5.5;  $7^{\circ}\text{C}$  and pH 6.0;  $7^{\circ}\text{C}$  and pH 6.3;  $12^{\circ}\text{C}$  and pH 5.0;  $12^{\circ}\text{C}$  and pH 6.0; and  $12^{\circ}\text{C}$  and pH 6.3) was monitored at 3, 6, 9, 12, 24, 30, 36, 48, 54, 60, 72, 84, 96, 108, and 120 h of incubation. At each interval, an aliquot of 1 mL was transferred to a tube containing 9 mL of 0.1% (w/v) peptone water, and was serially diluted. Subsequently, a 0.01-mL aliquot from appropriate dilutions was taken and plated on Trypticase Soy Agar (TSA) plates (HiMedia, Mumbai, India) by microdrop technique. The plates were incubated at  $28^{\circ}\text{C}$  for 24 h, and the colonies were counted.

### Analysis of growth data for obtaining models

The model for *P. fluorescens* growth in meat broth was developed using the two-stage methodology. In the first stage, the maximum specific growth rate ( $\mu_{\text{max}}$ ), and the lag phase ( $\lambda$ ) were calculated for each experimental combination. The growth parameters were obtained by the modified Gompertz and Baranyi and Roberts equation to the experimental data by using DMFit 3.0 program. In the second stage, the estimates obtained for  $\mu_{\text{max}}$  were adjusted for the extended Ratkowsky model to determine the effect of temperature and pH on the maximum specific growth rate of *P. fluorescens*, according to the following equation:

$$\mu_{\text{max}} = a(\text{pH} - \text{pH}_{\text{mim}})(T - T_{\text{mim}})^2 \quad (1)$$

where  $a$  is the regression constant, and  $\text{pH}_{\text{mim}}$ ,  $T_{\text{mim}}$  are the minimum pH and minimum temperature, respectively, estimated theoretically for the microbial growth.

### Validation of the results by statistical analysis of models

The following statistical parameters were calculated for the validation of the models: correlation coefficient ( $R^2$ ), mean square error (RMSE), bias factor, and accuracy factor.<sup>14</sup> The correlation coefficient ( $R^2$ ) describes the model fit throughout the length of the curve; the closer the value of  $R^2$  is to one, the better the model fit is.

The mean square error (RMSE) is given by Eq. (2), and presents the model error relative to the data, that is, how close are the predicted values to the observed values; where the closer to zero indicates a better fit.

$$\text{RMSE} = \frac{\text{SQR}}{n} = \sqrt{\Sigma(\text{value}_{\text{obs}} - \text{value}_{\text{pred}})^2} \quad (2)$$

where  $value_{obs}$  is the experimental value,  $value_{pred}$  is the value predicted by the model,  $SQR$  is the sum of square residuals, and  $n$  is the number of degrees of freedom (number of data points – number of model parameters).

The bias factor shown in Eq. (3) gives the same weight to the average values that over- or underestimates the average, that is, an average relative deviation.

$$\text{Bias factor} = 10^{\sum \log((value_{obs} - value_{pred})/n)} \quad (3)$$

where  $value_{obs}$  is the experimental value,  $value_{pred}$  is the value predicted by the model, and  $n$  is the number of data points minus the number of model parameters.

The accuracy factor is the most reliable and accurate statistical measurement, as it uses both, the predicted and observed values, and determines the percentage prediction error. This factor takes into account only the absolute values. The closer the value is to 1, the lower the percentage error is. The calculation factor is corrected by Eq. (4).

$$\text{Accuracy factor} = 10^{\sum |\log(value_{obs} - value_{pred})| / n} \quad (4)$$

where  $value_{obs}$  is the experimental value;  $value_{pred}$  is the value predicted by the model, and  $n$  is the number of data points minus the number of model parameters.

## Results and discussion

### Primary growth model of *P. fluorescens* in meat broth

Table 1 shows the growth parameters for *P. fluorescens* at three different temperatures and pH values.

The modeled growth parameters in Table 1 show that, in general, different pH values did not affect the growth of *P. fluorescens* at a given temperature. This is evident, for example, considering the concentration of  $5 \log \text{CFU mL}^{-1}$ , when the deterioration process starts. This concentration of population is reached in about 50 h at all temperatures, regardless of the pH value (Fig. 1).

The final meat pH can vary from 5.5 to 7.0, and is highly dependent on the amount of glycogen present in the tissue at the time of slaughter, since it turns into lactic acid

after slaughter.<sup>15</sup> The final pH value directly affects microbial growth,<sup>16</sup> as at low pH, weak acids in the undissociated form can pass through the plasma membrane of the microorganism. Then, the acid dissociates by consuming ATP, releasing protons, and stabilizing pH; this results in cell death.<sup>17</sup> On the other hand, when food is stored under aerobic conditions, the pH tends to increase, leading the total cell counts greater than  $10^7 \text{CFU mL}^{-2}$ . These changes are caused possibly due to the formation of basic substances synthesized mainly during the *Pseudomonas* growth.<sup>18</sup> However, this relationship between the increased microbial growth rate and higher pH values was observed only at  $12^\circ\text{C}$ . An earlier study investigated the microbiological deterioration of ground beef at a pH range from 5.34 to 6.13, and found a significant effect of meat pH on the growth kinetic parameters of *Pseudomonas*.<sup>19</sup> In contrast, some other studies<sup>16,20</sup> reported that, depending on the conditions, pH might not have effect on microbial growth in a range close to that employed in this work.

Moreover, it was observed that the increase in storage temperature from  $4^\circ\text{C}$  to  $12^\circ\text{C}$  led to a decrease in the lag phase and an increase in the exponential phase. This behavior led to a population concentration of about  $6 \log \text{CFU mL}^{-1}$  after 70, 40, and 24 h, at  $4^\circ\text{C}$ ,  $7^\circ\text{C}$ , and  $12^\circ\text{C}$ , respectively, independent of the pH value (Figs. 1–3).

A study investigated the prediction of shelf life of pork, and found that the total viable count reached  $6 \log \text{CFU mL}^{-1}$  after 25 h, 60 h, and 100 h at  $15^\circ\text{C}$ ,  $7^\circ\text{C}$ , and  $4^\circ\text{C}$ , respectively.<sup>21</sup>

As shown in Table 1, a higher lag phase was observed at  $7^\circ\text{C}$  and pH 5.5 by the adjustment of the two primary models to the experimental data. Moreover, the lag phase ( $\lambda$ ) was lower at the highest pH (6.3) tested, probably because most bacteria grow best in an environment having pH near to neutral (7.0). However, despite the growing conditions at  $7^\circ\text{C}$ , a higher lag phase was observed at more acidic pH (5.5), and the same was also observed for  $\mu_{max}$ . This behavior is opposite of that observed by other authors, as Gospavic et al.<sup>22</sup> and Koutsomanis et al.<sup>23</sup> found that at a higher lag phase, the maximum growth rate was lower. It is important to mention that these authors used a higher temperature range of  $0\text{--}15^\circ\text{C}$  and  $0\text{--}20^\circ\text{C}$ , compared to the one used in this work. It is noteworthy that the findings of Gospavic et al.<sup>22</sup> have shown that the growth of *Pseudomonas* spp. adjusted to the modified Gompertz model presented a

**Table 1 – Growth parameters of *P. fluorescens* in meat broth at  $4^\circ\text{C}$ ,  $7^\circ\text{C}$ , and  $12^\circ\text{C}$ , and pH 5.5, 6.0, and 6.3.**

Growing conditions	Baranyi and Roberts		Gompertz	
	$\lambda$ (h)	$\mu_{max}$ ( $\text{h}^{-1}$ )	$\lambda$ (h)	$\mu_{max}$ ( $\text{h}^{-1}$ )
$4^\circ\text{C}$ and pH 5.5	19.99	0.040149	–	0.030961
$4^\circ\text{C}$ and pH 6.0	18.64	0.043955	18.63	0.042155
$4^\circ\text{C}$ and pH 6.3	19.65	0.043430	20.59	0.042679
$7^\circ\text{C}$ and pH 5.5	20.86	0.082562	19.75	0.075270
$7^\circ\text{C}$ and pH 6.0	18.74	0.081530	16.43	0.070892
$7^\circ\text{C}$ and pH 6.3	14.89	0.071963	14.42	0.067418
$12^\circ\text{C}$ and pH 5.5	$8.27 \times 10^{-8}$	0.087592	–	0.070049
$12^\circ\text{C}$ and pH 6.0	$7.91 \times 10^{-8}$	0.092016	–	0.074098
$12^\circ\text{C}$ and pH 6.3	$1.43 \times 10^{-7}$	0.100424	–	0.086309

$\lambda$ , duration of lag phase;  $\mu_{max}$ , maximum specific growth rate.

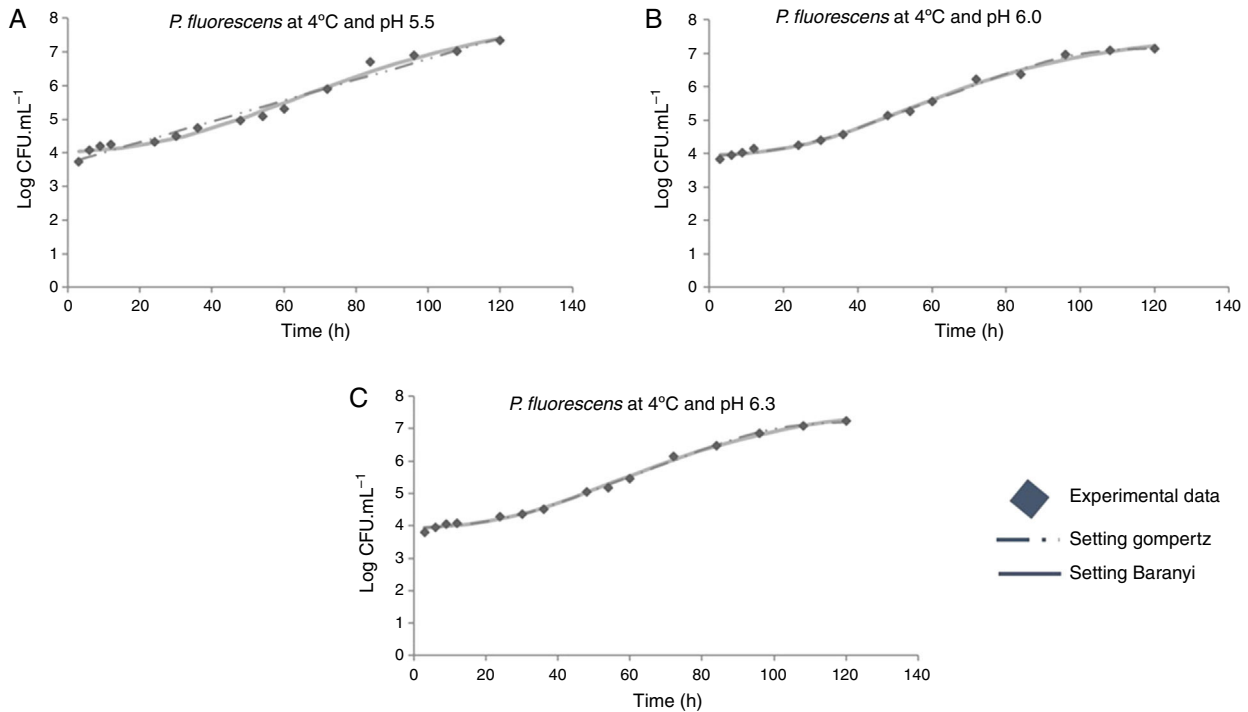


Fig. 1 – Primary growth modeling of *P. fluorescens* at 4 °C and pH 5.5 (A), pH 6.0 (B), and pH 6.3 (C).

higher lag phase at 2 °C and 4 °C, corroborating the increase in  $\mu_{max}$ . The decrease in  $\lambda$  and the increase in  $\mu_{max}$  become clear when the values at 2 °C and 4 °C are compared with those obtained at 15 °C and 20 °C. The maximum tested value in this work was 12 °C.

At 12 °C, the estimated  $\lambda$  values adjusted by the Baranyi and Roberts model to the experimental data were very low. In contrast, these values were not estimated by the modified Gompertz model, evidencing no lag phase at 12 °C.

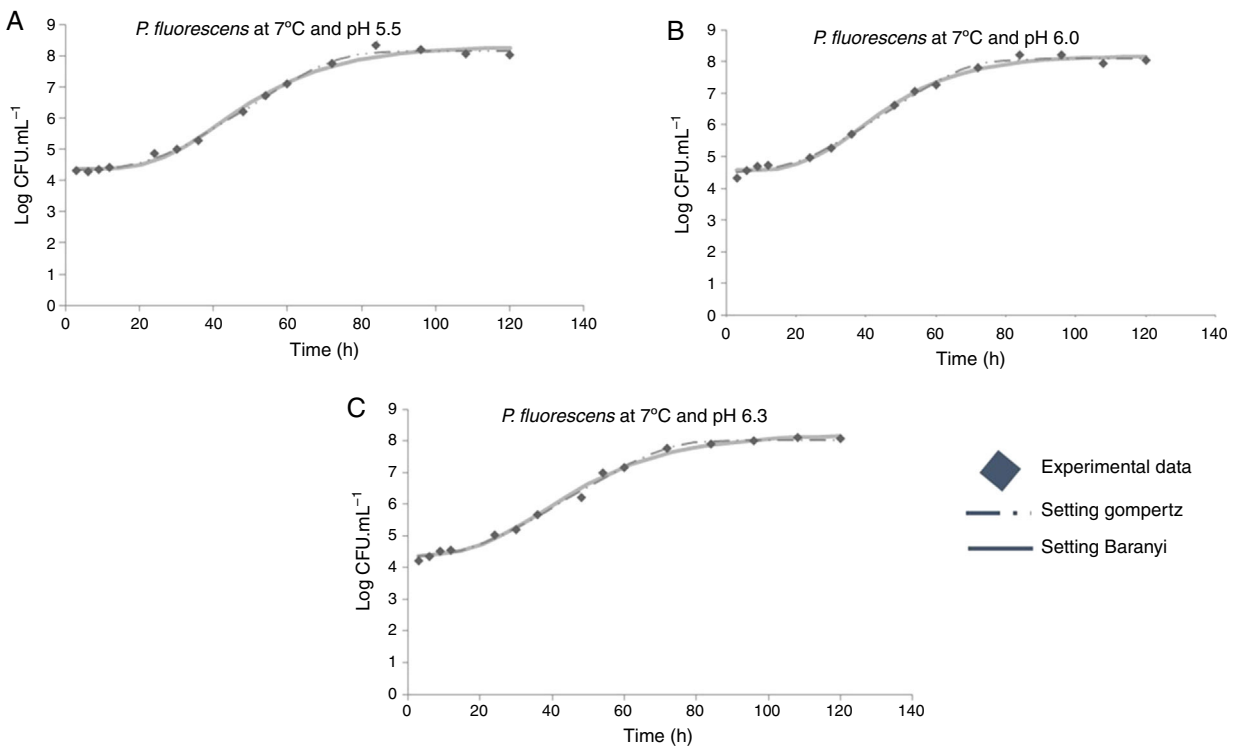


Fig. 2 – Primary growth model of *P. fluorescens* at 7 °C and pH 5.5 (A), pH 6.0 (B), and pH 6.3 (C).

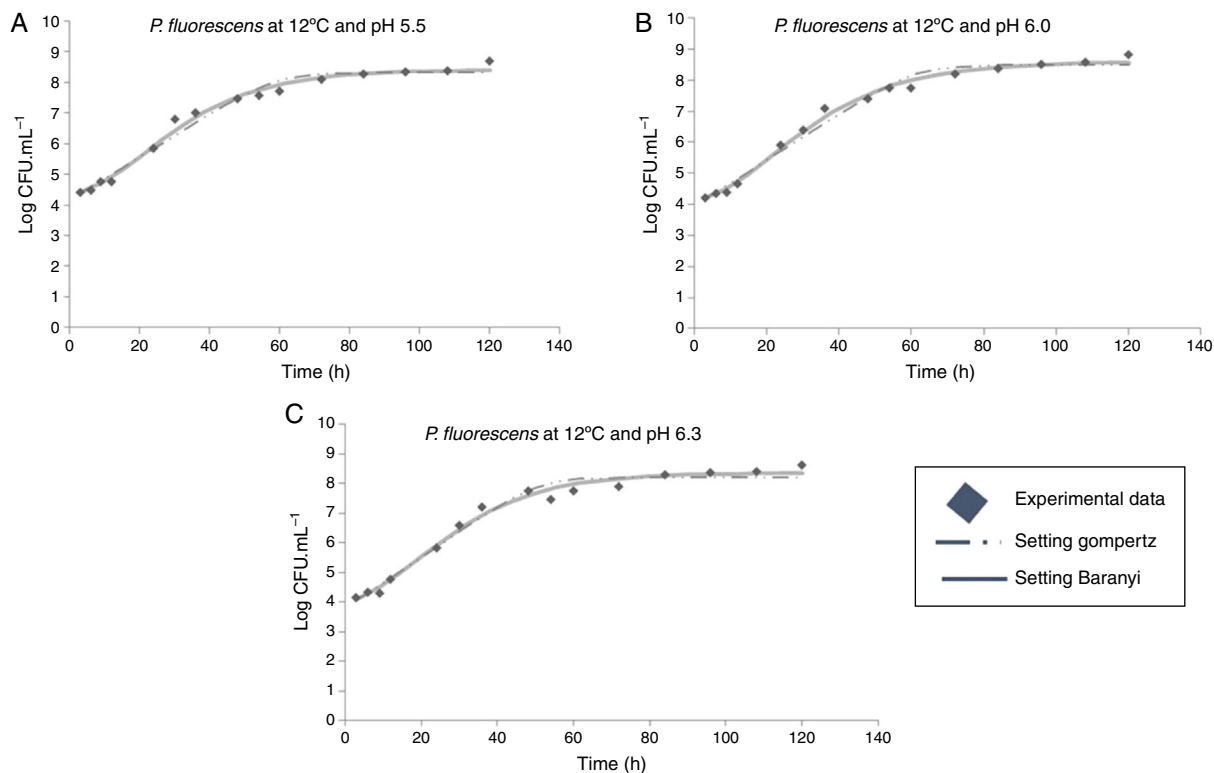


Fig. 3 – Primary growth model of *P. fluorescens* at 12 °C and pH 5.5 (A), pH 6.0 (B), and pH 6.3 (C).

With respect to the temperature increase from 7 °C to 12 °C, although a decrease in lag phase was observed, no significant changes were observed for the maximum specific growth rate, as can be seen in Table 1. At a greater range of growing conditions such as 4 °C and 12 °C, the  $\lambda$  values significantly decreased, while  $\mu_{max}$  increased considerably.

The modified Gompertz model could not estimate the  $\lambda$  value either for the growing condition at 4 °C and pH 5.5 or at 12 °C. As the *P. fluorescens* culture was activated before being inoculated in meat broth, the lag phase might have been suppressed. In addition, the Gompertz model does not consider the adaptation phase (lag phase), but only the increase in the number of cells within the exponential growth.<sup>15</sup> Therefore, in some growth conditions, this phase could not be estimated by this model.

In the combined effect of the pH and incubation temperature on *P. fluorescens* growth, in general, higher temperature and higher pH result in the higher specific growth rate but lower lag phase. Thus, meat with initial pH closer to neutral and stored under temperature abuse may have short shelf life.

Table 2 shows the coefficient of determination ( $R^2$ ) and the root mean square error (RMSE) for the primary models.

For all growing conditions shown in Table 2, the primary models showed good fit to the experimental data, with  $R^2$  values very close to 1; and low RMSE values demonstrated that the predicted values are close to the observed values, which ensure the validation of data. Gospavic et al.<sup>22</sup> modeled the growth of *Pseudomonas* spp. in poultry at different temperature conditions, and also found good fit to the data by the Baranyi and Roberts and the modified Gompertz models.

Table 2 –  $R^2$  and RMSE of primary growth model for *P. fluorescens*.

Growing conditions	Baranyi and Roberts		Gompertz	
	$R^2$	RMSE	$R^2$	RMSE
4 °C pH 5.5	0.98	0.1701	0.97	0.2105
4 °C pH 6.0	0.99	0.1026	0.99	0.0959
4 °C pH 6.3	0.99	0.8333	0.99	0.0854
7 °C pH 5.5	0.989	0.1703	0.996	0.1077
7 °C pH 6.0	0.990	0.1469	0.993	0.1284
7 °C pH 6.3	0.992	0.1400	0.994	0.1154
12 °C pH 5.5	0.987	0.1815	0.972	0.2612
12 °C pH 6.0	0.991	0.1614	0.979	0.2505
12 °C pH 6.3	0.984	0.2126	0.968	0.2967

**Table 3 – The Baranyi and Roberts and the modified Gompertz growth models for *P. fluorescens* at 4 °C, 7 °C and 12 °C, and pH 5.5, 6.0, and 6.3.**

Model	Growing conditions	Equation
Baranyi and Roberts	4 °C pH 5.5	$dN/dt = -2.23159 * 0.040149 * [1 - N(t)/7.412011] * N(t)$
	4 °C pH 6.0	$dN/dt = -2.2691713 * 0.043955 * [1 - N(t)/7.225329] * N(t)$
	4 °C pH 6.3	$dN/dt = -2.3479932 * 0.04343 * [1 - N(t)/7.281148] * N(t)$
Gompertz	4 °C pH 5.5	$\text{Log}N(t) = 3.797973 + 3.62246489\text{exp}\{-\text{exp}[-0.023233(t-43.04189)]\}$
	4 °C pH 6.0	$\text{Log}N(t) = 3.952039 + 3.19371571\text{exp}\{-\text{exp}[-0.03588(t-46.49816)]\}$
	4 °C pH 6.3	$\text{Log}N(t) = 3.952664 + 3.24166902\text{exp}\{-\text{exp}[-0.035789(t-48.53329)]\}$
Baranyi and Roberts	7 °C pH 5.5	$dN/dt = 5.5991153 * 0.082562 * [1 - N(t)/8.264927] * N(t)$
	7 °C pH 6.0	$dN/dt = -4.6091953 * 0.08153 * [1 - N(t)/8.163269] * N(t)$
	7 °C pH 6.3	$dN/dt = 2.9202683 * 0.071963 * [1 - N(t)/8.157786] * N(t)$
Gompertz	7 °C pH 5.5	$\text{Log}N(t) = 4.344648 + 3.8213554\text{exp}\{-\text{exp}[-0.053543(t-38.4268)]\}$
	7 °C pH 6.0	$\text{Log}N(t) = 4.523484 + 3.56886414\text{exp}\{-\text{exp}[-0.053996(t-34.94508)]\}$
	7 °C pH 6.3	$\text{Log}N(t) = 4.361284 + 3.66777251\text{exp}\{-\text{exp}[-0.49965(t-34.4322)]\}$
Baranyi and Roberts	12 °C pH 5.5	$dN/dt = -1 * 0.087592 * [1 - N(t)/7.25E-09] * N(t)$
	12 °C pH 6.0	$dN/dt = -1 * 0.092016 * [1 - N(t)/7.28E-09] * N(t)$
	12 °C pH 6.3	$dN/dt = -1 * 0.0100424 * [1 - N(t)/1.44E-08] * N(t)$
Gompertz	12 °C pH 5.5	$\text{Log}N(t) = 3.79214 + 3.93009306\text{exp}\{-\text{exp}[-0.04845(t-20.63974)]\}$
	12 °C pH 6.0	$\text{Log}N(t) = 4.17734 + 4.31045923\text{exp}\{-\text{exp}[0.046728(t-21.40046)]\}$
	12 °C pH 6.3	$\text{Log}N(t) = 4.053301 + 4.15074063\text{exp}\{-\text{exp}0.056523(t-17.69189)]\}$

Table 3 shows the Baranyi and Roberts and the modified Gompertz models for *P. fluorescens* at 4 °C, 7 °C, and 12 °C, and pH 5.5, 6.0, and 6.3.

Once validated, the equations (Table 3) can be used for prediction of *P. fluorescens* growth in meat broth under the conditions tested.

Therefore, for the growing conditions employed in this study, it is possible to assess the cell concentration rate over time using the equations generated in the Baranyi and Roberts models. In addition, it is also possible to assess the cell concentration at any time by the equations generated by the modified Gompertz model.

Many studies have used combination of the sensory evaluation and the predictive microbiology to estimate the shelf life of a particular food. Kreyenschmidt et al.<sup>24</sup> and Nychas et al.<sup>25</sup> estimated, by sensory parameters, the shelf life of poultry infected by *Pseudomonas* spp. when microbial population reached 7 log CFU g<sup>-1</sup> using the prediction curves obtained by the primary models. Then, the authors calculated the time to achieve a growth rate of 7 log CFU g<sup>-1</sup> under the environmental conditions tested, which was considered the end of shelf life for the meat. Therefore, the predictive models can also be used for the estimation of shelf life of food products.

**Secondary growth model for *P. fluorescens* in meat broth**

Table 4 shows the secondary models generated for the effect of temperature and pH on the maximum specific growth rate.

**Table 4 – Secondary models for *P. fluorescens*.**

Primary model	Secondary model of the square root
Baranyi and Roberts	$\mu_{\max} = 0.0000267 \times [(T - (-14.6619))^2] \times (\text{pH} - 0.707962)$
Gompertz	$\mu_{\max} = 0.00000224 \times [(T - (-64.1401))^2] \times (\text{pH} - 0.631746)$

**Table 5 – Statistical parameters, R<sup>2</sup>, RMSE, bias factor, and accuracy factor for validation secondary modeling of *P. fluorescens*.**

Models	R <sup>2</sup>	RMSE	Bias factor	Accuracy factor
1	0.7134	0.0243	0.9990	1.6233
2	0.2310	0.0351	0.9781	2.6231

Model 1 is the square root model generated from Baranyi and Roberts model, and Model 2 is the square root model generated from the modified Gompertz model.

The statistical parameters for the validation of the secondary model for the growth data are shown in Table 5.

Considering the data in Table 3, a secondary Ratkowsky model was used to evaluate the effect of pH and incubation temperature on *P. fluorescens* growth, which was best validated from primary data ( $\mu_{\max}$ ) generated by the Baranyi and Roberts model, since it has smaller RMSE, and R<sup>2</sup>, and the bias factor and accuracy factor are close to 1.

**Conclusion**

The primary models generated showed a good fit to the experimental data and can be used to predict the growth of *P. fluorescens* under the conditions employed in this study. The primary modeling showed that the increase or decrease in pH had a little effect on the microbial growth parameters, when compared with the temperature conditions. With respect to the combined effect of temperature and pH, in general, the increase in temperature concomitant with higher pH led to an increase in  $\mu_{\max}$  and a decrease in  $\lambda$  value, resulting in a shorter shelf life, especially considering the temperature abuse tested.

The secondary modeling using the Ratkowsky model obtained from the data of the Baranyi model was validated

better than that obtained by the Gompertz model, especially because the latter showed a low goodness of fit indices.

Thus, the equation in the secondary model obtained from the Baranyi model can be used to predict the effect of pH and temperature on *P. fluorescens* growth.

### Conflicts of interest

The authors declare no conflicts of interest.

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