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4
1

Statistical evaluation of mathematical models for *Salmonella typhimurium* growth

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ABSTRACT
Food illness is a serious health threat and has significant economic consequences for people in both the developing and developed world. *Salmonella* genus is one of the most common pathogens and a major cause of foodborne illness in humans worldwide. Nowadays, the application of mathematical models and functions to describe the microorganism growth kinetics provides a new behavioral vision of the interaction between microorganisms and the environment. Lately the studies on the subject have been gathering interest in the elaboration and application of mathematical modeling and equations over the last years to be used in biotechnological and industrial process, therefore being a most useful tool, with the intent of reducing time and expenses associated with the conventional tests. The purpose of the present study was to compare the Baranyi and Roberts (1994) model with quadratic function generated from data experimentally obtained of the Salmonella *typhimurium* growth in vitro. It was observed that the quadratic function had a better fitting to describe the kinetics growth of Salmonella *typhimurium*, this function being a low cost, efficient and easily applied tool.

Keywords: microbial growth; *Salmonella sp*; mathematical models.

Introdução

It was estimated that foodborne diseases cause approximately 76 million illnesses, 325.000 hospitalizations, and 5.000 deaths in the United States each year. Salmonellosis is a most prevalent foodborne disease in many countries world-wide and it has been estimated that approximately 1 million cases were reported annually in the developed nations such as USA (MEAD et al., 1999; FDA, 2001; CDC, 2010).

Traditionally, the microbiological safety of foods has been established by challenge tests. These tests simulate the effects of environmental conditions on food, in terms of growth and proliferation of spoilage and pathogenic microorganisms. Challenge tests can provide data useful in determining the safety and shelf-life of food under set conditions, for the effect of temperature, pH, water activity and nitrite concentration (McDONALD, SUN, 1999).

Mathematical modeling of microbial growth has been used to estimate parameters (specific growth rate and lag time) required to study growth under different physical and chemical conditions, to enable the effects of antimicrobials to be investigated, to formulate appropriate microbiological media or to build up prediction models for use in food and fermentation microbiology (LOPEZ et al., 2004). Several approaches to modeling bacterial growth can be found in the microbiological literature (BARANYI, ROBERTS, McCLURE, 1993; PERNI, ANDREW, SHAMA, 2005).

This modeling can be applied at various levels. A primary level model is an equation or function that is used to describe the microbial response over time with a characteristic set of parameter values.

Microbial response has been mostly expressed in terms of microbial numbers (concentration of colony forming units) or optical density as an indirect measurement (LOPEZ et al., 2004). The mathematical modeling of the evolution of microorganisms is an important step in quantitatively describing the influence of processing conditions on food safety (VAN IMPE et al., 2005; LENHARD, CARRIER, 2017, next to the mathematical modeling of quality influencing factors.

The term "model" has been defined in several reviews on the mathematical modeling of microbiological processes. A purely empirical model, like the quadratic response surface for the environment dependence of a parameter of a bacterial population is also a model, but its aim is nothing more than a smooth representation of the experimental results. It is a regression model in the sense used in the regression analysis of statistics where the aim is the numerical representation of certain responses by means of simple functions, like polynomials, without mechanistic explanation. The term *mathematical* model is more rigorous and refers to a set of basic hypotheses on the studied processes, some of which are possibly expressed by means of functions and equations. Therefore, from a mechanistic point of view, function and model are not equivalent terms. Function is a mathematical abstraction making it easier to describe a particular model (BARANYI, ROBERTS, 1995).

Selecting the most appropriate growth model is often a matter of trial and error. Moreover, there are different criteria for determining the suitability of one particular model over another vary: some authors have relied on mathematical measures for a good fitting while others have focused on direct comparisons of particular growth parameters as predicted by the various models (PERNI, ANDREW, SHAMA, 2005).

There are a number of sigmoid functions that have been used for modeling somatic growth and population dynamics, which could be applied to microbial growth. Despite, the number of different nonlinear equations used as growth functions, there is not one growth function that is essentially superior to all others. Although there has been considerable effort to derive alternative models for microbial growth, and there are some comparative studies reported in the literature, it is necessary to investigate their statistical ability to fit experimental data (LOPEZ et al., 2004).

The purpose of the present study was to compare the Baranyi and Roberts (1994) model with quadratic function generated from data experimentally obtained of the *Salmonella typhimurium* growth *in vitro* over time.

Material e Métodos

1. Test organism

Lyophilized culture of *Salmonella typhimurium* ATCC 14028, strain obtained from Fundação André Tosello's collection, hydrated with 500μL of sterile saline (0,85% NaCl). The stock culture was grown for 24h at 35°C on Brain Heart Infusion (BHI) broth (Merck) followed by streaking on BHI agar (Merck) slants and incubated at 35°C ± 1 for 24h.

2. Procedures

Preparation of inoculum

Salmonella typhimurium inoculum was prepared starting from suspension in 4mL of sterile saline and 1μL of the *Salmonella* suspension was then inoculated into 500mL of BHI. This was incubated for at 35°C ± 1 during the experiment.

Kinetic growth of *Salmonella typhimurium*

The microorganism kinetics was established based on the methodologies of Gupta, Sharma, Vyas (1995) and López et al. (2004). Portions of 1mL the inoculum were submited to determine absorbance at 600nm using a spectrophotometer (B582- Micronal) in intervals of 30 minutes or 1 hour, depending on the microrganisms kinetics growth up to 24 hours. In parallel, serial dilutions of each *Salmonella* culture were prepared, using a set of 11 tubes that contained each 9 mL sterile saline and were homogenized in rotary shaker (Quimis) for 5 seconds. The bacterial concentrations were also determined by pour plating 1mL of samples on BHI agar in duplicate. Plates were incubated at 35°C for 24 h and enumerated, microbial counts were reported as CFU/mL. The growth curve was determined by plotting the logarithm of the relative population size versus time. Growth data were fit by each mathematical model in this study.

Mathematical procedure and statistical analysis

Viable cell counts were converted to logarithmic and the growth curve of *Salmonella typhimurium* were analyzed by the Baranyi and Roberts (1994) model (Equation1) and by quadratic function (Equation 2) on a computer using the Statistica 6.1 (Statsoft, 1997) package.

Below Baranyi and Roberts (1994) model:

$$y(t) = y_0 + \mu_{\max} t + \frac{1}{\mu_{\max}} \ln\left(e^{-vt} + e^{-h_0} - e^{-vt-h_0}\right)$$

$$- \frac{1}{m} \ln\left(1 + \frac{\left(e^{m\mu_{\max} t + \frac{1}{\mu_{\max}} \ln\left(e^{-vt} + e^{-h_0} - e^{-vt-h_0}\right)}\right) - 1}{e^{m(y_{\max} - y_0)}}\right)$$

Equation (1)

Where:

- $y(t) = \ln(x(t))$ with $x(t)$ the cell concentration (cfu/mL)
- $(y_0) = \ln(x_0), y_{\max} = \ln(x_{\max}), x_0$ being the initial and $x_{\max}$ the asymptotic cell concentration, respectively.
- $\mu_{\max}$ is the maximum specific growth rate (1/h)
- m is a curvature parameter to characterize the transition from the exponential phase
- v is a curvature parameter to characterize the transition to the exponential phase
- h_0 is a dimensionless parameter quantifying the initial physiological state of the cells. From that, the lag time $\lambda\,(h)$ can be calculated as $h_0 / \mu_{\max}$

Quadratic function (Silva et al., 2002)

$$y(t) = \beta_2 t^2 + \beta_1 t + \beta_0 \qquad \text{Equation (2)}$$

Where β_0, β_1 and β_2 are constant regression coefficients; t = time (hours); y = microbial biomass (CFU/ml); CFU = Colony Forming Units.

The *F-test* was used as a criterion to validate the Baranyi and Roberts (1995) model and Quadratic function was the level of significance of the Baranyi and Roberts (1994) model and quadratic function were established comparing the listed F-values and the calculated F-values for each operating condition.

Resultados e Discussão

Kinetics growth of *Salmonella typhimurium*

Optical density (absorbance) and viable count data used to build de kinetic of *Salmonella typhimurium* ATCC 14028 are illustrated in Table 1.

Basti and Razavilar (2004) in their experiments obtained a cell concentration of 2 x 10^7 CFU/mL for the culture of *Salmonella typhimurium* with Brain Heart Infusion broth (BHI), reading in optical density (OD) of 600nm and absorbance of 0.03. Similar results 2.5 x 10^7 CFU/mL were obtained in this research in the time of 7 hours, in the same conditions.

In another study executed by Cogan (2001) with *Salmonella enteritidis* utilizing broths that were standardised to an optical density of 0,2 at 600nm to give 2 x 10^8 CFU/mL. The same results were obtained in this research (2 x 10^8 CFU/mL) in the same conditions.

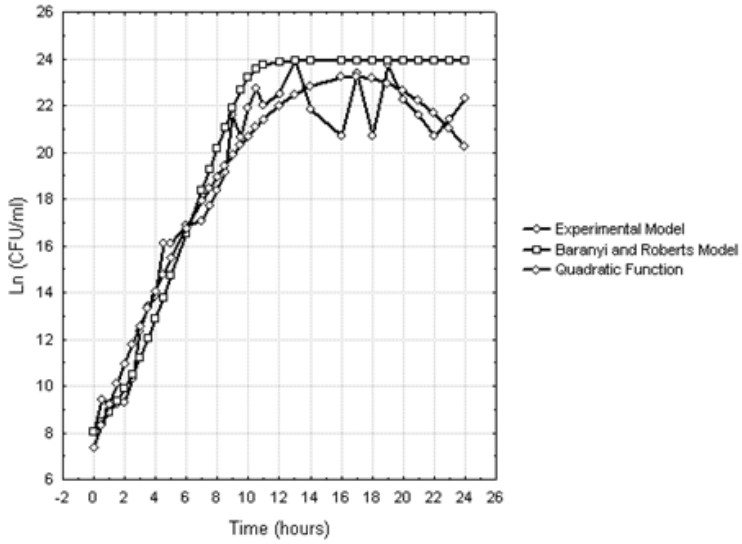
The study of the kinetics of the *Salmonella typhimurium* ATCC 14028 in 24 hours enabled the construction of the growth curve seen in Figure 1 (experimental model). The same figure shows the curves constructed by Baranyi and Roberts model (1994) and quadratic function.

Table 1. Optical density and viable count of *Salmonella typhimurium* ATCC 14028 in the different phases of monitored growth for 24 hours.

Time(hours)		Optical density (OD)		Count (CFU/mL)	
Phases of Growth		Initial	Final	Initial	Final
0 a 2h30m	Phase Lag	-0.001	0.001	$3.0x10^3$	$3.0x10^4$
3 a 10h30m	Phase Log	0.002	0.805	$2.3x10^5$	$3.2x10^9$
11h a 24h	Phase Stationary	0.797	1.114	$3.7x10^9$	$5.0x10^9$

CFU/mL- CFU = Colony Forming Units/ milliliter.

Figure 1. Fitting of experimental data obtained in the growth of *Salmonella typhimurium* ATCC 14028 to the Baranyi and Roberts model (1994) and quadratic function.



Tables 2 and 3 shows that Fcalculated was higher than Ftabled and that the determination coefficient (R^2) was superior to 0.9, that is, the fitting of experimental data to the Baranyi and Roberts model (1994) and quadratic function were significant ($p<0.05$). Table 4 shows the quadratic equation regression coefficients that describe the present research phenomenon.

On Table 2 and 3 the values of determination coefficient (R^2) in the regression analysis was 0.93 and 0.95, respectively. According to Vieira (2004) it is necessary the analysis of residues in order to analyze the fitting of the experimental data to the model besides the variance analysis.

Table 2. Variance analysis of the fitting of the experimental data to the Baranyi and Roberts model (1994).

Source	SS	DF	MS	F_c	F_t
Regression	1178.1088	2	589.0544	201.63	3.32
Residue	87.6465	30	2.9215		
Total	1265.7553	32		R^2=0.93	p<0,05

SS: sun square; DF: degree of freedom; MS: mean square; F_c : F calculated; F_t : F tabulated.

Table 3. Variance analysis of the fitting of the experimental data to the Quadratic function.

Source	SS	DF	MS	F_c	F_t
Regression	791.2097	2	395.6048	289.00	3.32
Residue	41.0657	30	1.3689		
Total	832.2754	32		R^2=0.95	p<0,05

SS: sun square; DF: degree of freedom; MS: mean square; F_c: F calculated; F_t : F tabulated.

Table 4. Values of the second – order polynomial regression coefficients.

Coeficients		
β_2 *	β_1 *	β_0 *
-0,06	1,90	7,37

*significant (p<0,05)

According to Vieira (2004) when the fitting is satisfactory the graphic of the residues do not show tendencies and are dispersed in the range of ± 3, that being observed in the fitting of the responses to the quadratic function (Figure 2), differently of the graphic of residues generated by Baranyi and Roberts model (1994) that showed an apparent tendency, having 3 points outside the range of ± 3.

Figure 2 – Distribution of the obtained residues in the fitting of the experimental data to a quadratic function.

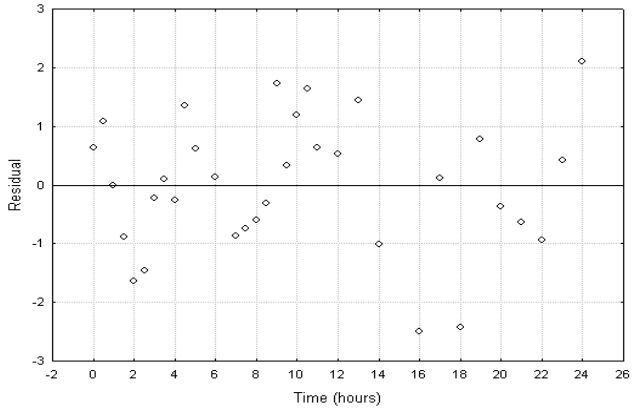


Figure 3 – Distribution of the obtained residues in the fitting of the experimental data to a Baranyi and Roberts model (1994).

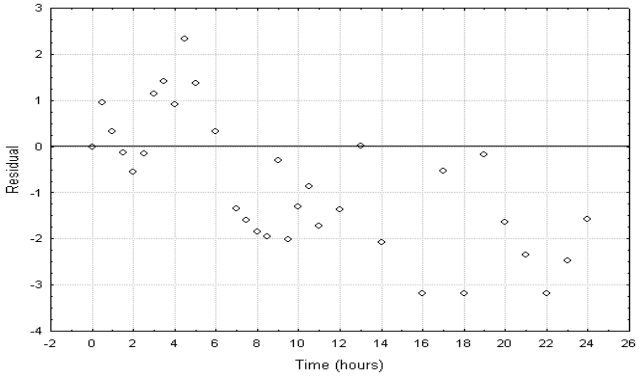


Figure 1 shows similarity between curve generated by the experimental data and Baranyi and Roberts mathematical modeling (1994) and quadratic function. However a difference was observed in the adaptation phase or lag phase. That was only seen in experimental modeling. The mathematical modeling shows linear and immediate growth from lag phase until the end of the exponential phase. This difference can be justified once the mathematical modeling is simplified and does not consider limitation factors for microbial growth culture, incubation, temperature, adaptation time, oxygen concentration, toxic metabolic concentration and biological space (BARANYI, ROBERTS, 1995; TRABULSI, ALTERTHUM, 2015).

The stationary phase in the experimental modeling varied up to a logarithmic unit, showing that even in a so-called stationary phase the microorganism keeps its adaptation capacity, with enables cellular multiplication even in adverse environment conditions. Figure 1 shows that the same could not be observed in the mathematical modeling.

As the growth rate depends on the substrate concentration the reduction of the nutrients availability during the exponential phase leads to a decline when the substrate is consumed. In this experiment, the stationary phase was established between to 11 to 24 hours (Table 1). The oscillation observed in stationary phase is justified by the alternative use of another source of energy for the maintenance of the microbial cell. Internal nutrients reserves of intermediate metabolic and the own structures of the microorganisms may act as source energy, for the respiratory activity and thus keep the bacteria viable for a considerable amount of time (SCHLEGEL, 1997; ROBINSON et al., 2001; TORTORA, FUNKE, CASE, 2017). However a self-limiting process mainly due to either the exhaustion of one of the essential nutrients and/or the accumulation of metabolic waste products, that may inhibit cell growth (VAN IMPE, 2005).

The main contribution of this paper is the utilization of the quadratic function of predictive microbial growth which reflect microbiological phenomena governing the *Salmonella typhimurium* growth this process. This research particularly focuses on the transition from the exponential growth phase to the stationary phase, which is induced through an increasing toxic product accumulation and/or substrate exhaustion.

However between two models obtained in this study, the quadratic function gave a better prediction (R^2 = 0.95) than the Baranyi and Roberts model in 1994 (R^2 = 0.93). The models produced in this study can be a good tool for a more cost effective evaluation of *Salmonella typhimurium* changes along of the production in food chain.

According Lenhard and Carrier (2017), although the use of mathematical models is ubiquitous in modern science, the involvement of mathematical modeling in the sciences is rarely seen as cases of interdisciplinary research. Often, mathematics is "applied" in the sciences, but mathematics also features in open-ended, truly interdisciplinary collaborations, as was observed in this paper as a response tool to minimize in vivo

experimentation in respect to animal ethics, standard biological model for analytical assays.

Conclusões

After the residues analyzes it was observed that the quadratic function showed a better fitting (R^2=0,95) in relation the Baranyi and Roberts (1994) with R^2= 0.93.

The developed quadratic function can be used to simulate the *Salmonella typhimurium* growth mode curve in the experimental conditions of the present research.

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