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MODELING AND PREDICTING THE BIOFILM FORMATION OF *SALMONELLA* VIRCHOW WITH RESPECT TO TEMPERATURE AND pH

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Biofilm formation of *Salmonella* Virchow was monitored with respect to time at three different temperature (20, 25 and 27.5 °C) and pH (5.2, 5.9 and 6.6) values. As the temperature increased at a constant pH level, biofilm formation decreased while as the pH level increased at a constant temperature, biofilm formation increased. Modified Gompertz equation with high adjusted determination coefficient (R^2_{adj}) and low mean square error (MSE) values produced reasonable fits for the biofilm formation under all conditions. Parameters of the modified Gompertz equation could be described in terms of temperature and pH by use of a second order polynomial function. In general, as temperature increased maximum biofilm quantity, maximum biofilm formation rate and time of acceleration of biofilm formation decreased; whereas, as pH increased; maximum biofilm quantity, maximum biofilm formation rate and time of acceleration of biofilm formation increased. Two temperature (23 and 26 °C) and pH (5.3 and 6.3) values were used up to 24 h to predict the biofilm formation of *S.* Virchow. Although the predictions did not perfectly match with the data, reasonable estimates were obtained. In principle, modeling and predicting the biofilm formation of different microorganisms on different surfaces under various conditions could be possible.

Keywords: Biofilm – modeling – pH – *Salmonella* Virchow – temperature

INTRODUCTION

Salmonella is one of the most common and widely distributed bacterial pathogens worldwide and the creative factor of salmonellosis [2]. It is a major public health problem and every year millions of human cases are reported worldwide. More than 95% of cases of infections caused by these bacteria are foodborne and these infections account for about 30% of deaths resulting from foodborne illnesses [13]. Studies have shown that these bacteria are capable of adhering and forming biofilms on different surfaces such as stainless steel, polymer and glass as well as biotic surfaces such as parsley, cantaloup, alfalfa etc. [1, 4, 14–15, 20, 27–29].

Adhesion of *Salmonella* to food surfaces was first reported by Duguid et al. [7]. Since that time, a number of documents have described the ability of foodborne pathogens to attach to food and food-contact surfaces, including *Listeria monocy-*

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togenes [8, 12, 18], *Yersinia enterocolitica* [12], *Campylobacter jejuni* [17] and *Escherichia coli* O157:H7 [5]. The attachment of pathogenic microorganisms to food-contact surfaces can lead to potential hygienic problems because pathogenic biofilms provide a reservoir of contamination. There is no doubt that biofilms containing pathogens increase the risk of microbial contamination in food plants [30]. Moreover, bacteria in biofilms exhibit enhanced resistance to cleaning and sanitation [10, 15, 24].

Environmental conditions such as temperature and pH play an important role in the phenotypic change from planktonic cells to the sessile form [30]. It has been demonstrated that biofilm formation by *Listeria* spp., *Salmonella* spp. and *Staphylococcus aureus* was greatly affected by temperatures ranging from 4 to 45 °C [11–12, 19, 22–23, 31]. Increase of biofilm formation with elevated temperatures has been reported [25, 31] as well as sub-optimal growing temperatures appeared to enhance biofilm production [25]. In some studies, effect of pH on biofilm formation also reported. *Pseudomonas fragi* showed maximum adhesion to stainless steel surfaces at pH ranges of 7–8, which is optimal for its cell metabolism [34], while other studies demonstrated that biofilm formation of *L. monocytogenes*, *Serratia liquefaciens, Shigella boydii*, *S. aureus*, *S. enteritidis*, and *Bacillus cereus* was induced at acidic conditions [21, 25, 39].

The objective of this study was (i) to describe the biofilm formation of *S.* Virchow with respect to temperature and pH by using primary and secondary models and (ii) predict biofilm formation at different temperature and pH values.

MATERIALS AND METHODS

Strains and culture conditions

A strain of *Salmonella enterica* serotype Virchow was used as a test organism provided by Prof. Dr. Mustafa Akçelik, Department of Biology, Ankara University. The strain was stored at –80 °C in Luria Bertani (LB) broth (Merck, Darmstadt, Germany) plus 80% (v/v) glycerol. The culture was then inoculated into LB broth and incubated at 37 °C for 18 h with shaking at 200 rpm. Inoculation was repeated twice.

Quantification of biofilm formation

Quantification of biofilm production in plastic microtitre plates was based on the method previously described by Vestby et al. [39] and Stepanovic et al. [36] with some modifications. In brief, the wells of a sterile 96 well flat bottom polystyrene microtiter plate were filled with 30 µL of overnight bacterial culture (10^8 cfu/mL) and 100 µL of the LB broth without NaCl (previously adjusted at pH 5.2, pH 5.9 and pH 6.6). *S.* Typhimurium LT2 strain was used as a control strain. The negative control wells contained broth only. The plates were incubated aerobically for different time

periods among 12 h to 96 h (in every 12 hours) at 20, 25 and 27.5 °C for modeling the biofilm formation. Furthermore, to predict the biofilm formation, plates contained LB without NaCl at pH 5.3 and pH 6.3 were also incubated at 23 and 26 °C for 24 h. The content of the plate was then poured off and the wells washed three times with 1% phosphate buffered saline. The remaining attached bacteria were fixed with 130 µL of 98% methanol (Merck, Germany) and after 10 min, microtiter plates were emptied and air dried. The microtiter plates were stained with 130 µL of Crystal violet (Merck, Germany) for 30 min. Excess stain was rinsed off by washing with distilled water. After, the microtiter plates were dried, the dye bound to the adherent cells was resolubilized with 130 µL of 33% (v/v) glacial acetic acid (Sigma-Aldrich, Steinheim, Germany) per well. The optical density (O.D.) of each well was measured at 595 nm using ELISA (Biorad, USA) reader.

Statistical analysis

Three independent trials were conducted for all the experiments, and means and standard deviations were calculated. One-way analysis of variance and Tukey's one-way multiple comparisons were used to determine significant differences of biofilm formations between different time levels. Level of significance was set to 0.05.

The biofilm formation modeling

Primary model

Modified Gompertz equation [Eq. (1)] [41–42] was used to describe the biofilm formation (OD_{595} vs. time) of *S.* Virchow:

$$y(t) = a \cdot \exp\left\{-\exp\left[\frac{\mu_m \cdot e}{a}(\lambda - t) + 1\right]\right\} \quad (1)$$

where t is time; $y(t)$ is OD_{595}; e is exp (1); a is asymptotic value, i.e. maximum biofilm concentration value reached; μ_m is the maximum biofilm formation rate and λ is the time of acceleration of biofilm formation.

Secondary model

Dependence of primary model parameters on temperature (T) and pH (pH) were described by the following equation:

$$a(T, pH) \text{ or } \mu_m(T, pH) \text{ or } \lambda(T, pH) = c_0 + c_1 T + c_2 T^2 + c_3 pH + c_4 pH^2 + c_5 TpH \quad (2)$$

where c_0, c_1, c_2, c_3, c_4 and c_5 are the coefficients of Eq. (2).

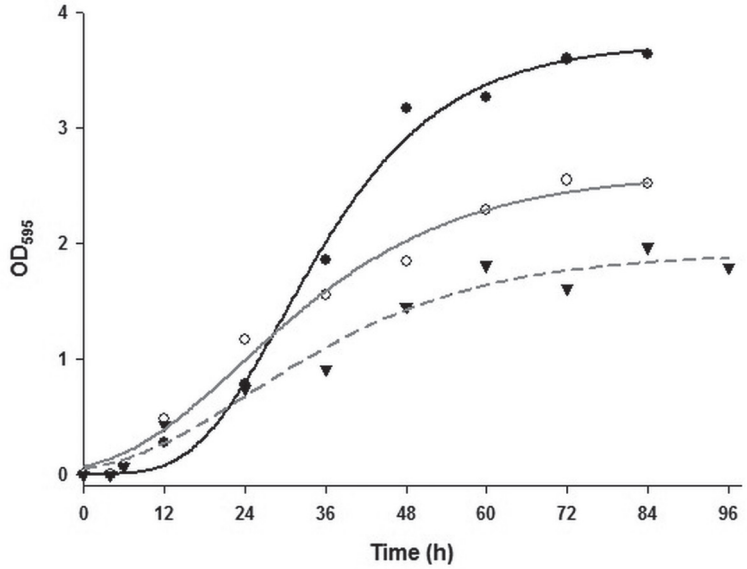

Fig. 1. Fit of modified Gompertz equation [Eq. (1)] to biofilm formation data of *Salmonella* Virchow at a constant pH (6.6) AT 20 °C (closed circles), 25 °C (open circles) and 27.5 °C (reverse closed triangle)

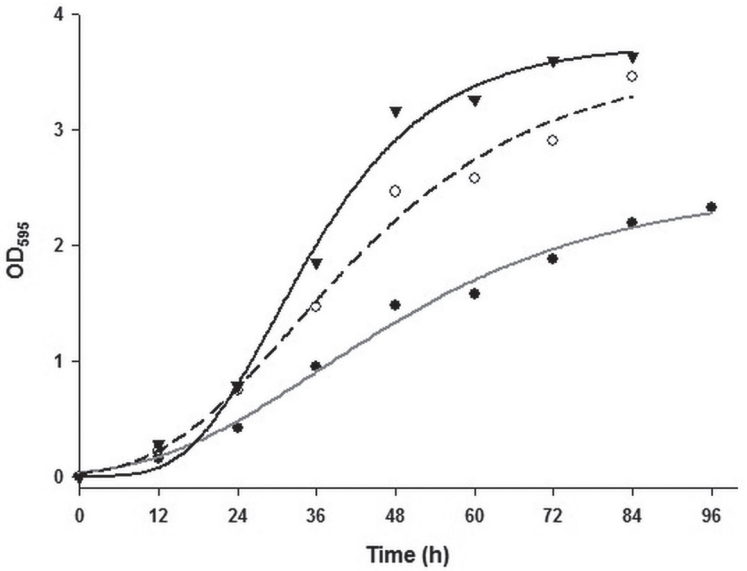

Fig. 2. Fit of modified Gompertz equation [Eq. (1)] to biofilm formation data of *Salmonella* Virchow at a constant temperature (20 °C) at 5.2 (closed circles), 5.9 (open circles) and pH 6.6 (reverse closed triangle)

Backward regression procedure was applied to remove the coefficients that are not significant ($P > 0.05$). Adjusted determination coefficient (R^2_{adj}) and mean square error (MSE) values were used to investigate the goodness-of-fit of the models. The parameters of the models were obtained by using SigmaPlot 2000 version 12.00 (Chicago, USA).

RESULTS

Effect of temperature and pH on biofilm formation

Biofilm formation of *S.* Virchow was monitored with respect to time at three different temperatures (20, 25 and 27.5 °C) and pH (5.2, 5.9 and 6.6) values. As the temperature increased from 20 to 27.5 °C at a constant pH level, biofilm formation decreased (Fig. 1). It was observed that when temperature was 30 °C or higher, very low biofilm formation or no biofilm formation occurred (data not shown). As the pH level increased from 5.2 to 6.6 at a constant temperature, biofilm formation also increased (Fig. 2). Significant ($p < 0.05$) increases in biofilm formation were observed after 12 h for each temperature and pH levels.

Modeling the biofilm formation

Visual inspection of the Fig. 1 and Fig. 2 indicated that the modified Gompertz equation [Eq. (1)] was adequate to describe the biofilm formation of *S.* Virchow. Table 1 shows R^2_{adj} and MSE values of each fit.

Secondary model fits (3-dimensional surfaces) were shown in Fig. 3. In general, as temperature increased maximum biofilm quantity (a), maximum biofilm formation rate (μ_m) and time of acceleration of biofilm formation (λ) decreased; whereas, as pH increased maximum biofilm quantity (a), maximum biofilm formation rate (μ_m) and

Table 1
Adjusted determination coefficient (R^2_{adj}) and mean square error (MSE) values for the fit of Eq. (1) to biofilm formation of *Salmonella* Virchow at three different temperature and pH values

Temperature (°C)	pH	R^2_{adj}	MSE
20	5.2	0.99	0.009
25		0.99	0.009
27.5		0.90	0.040
20	5.9	0.98	0.030
25		0.98	0.020
27.5		0.95	0.020
20	6.6	0.99	0.030
25		0.98	0.020
27.5		0.97	0.020

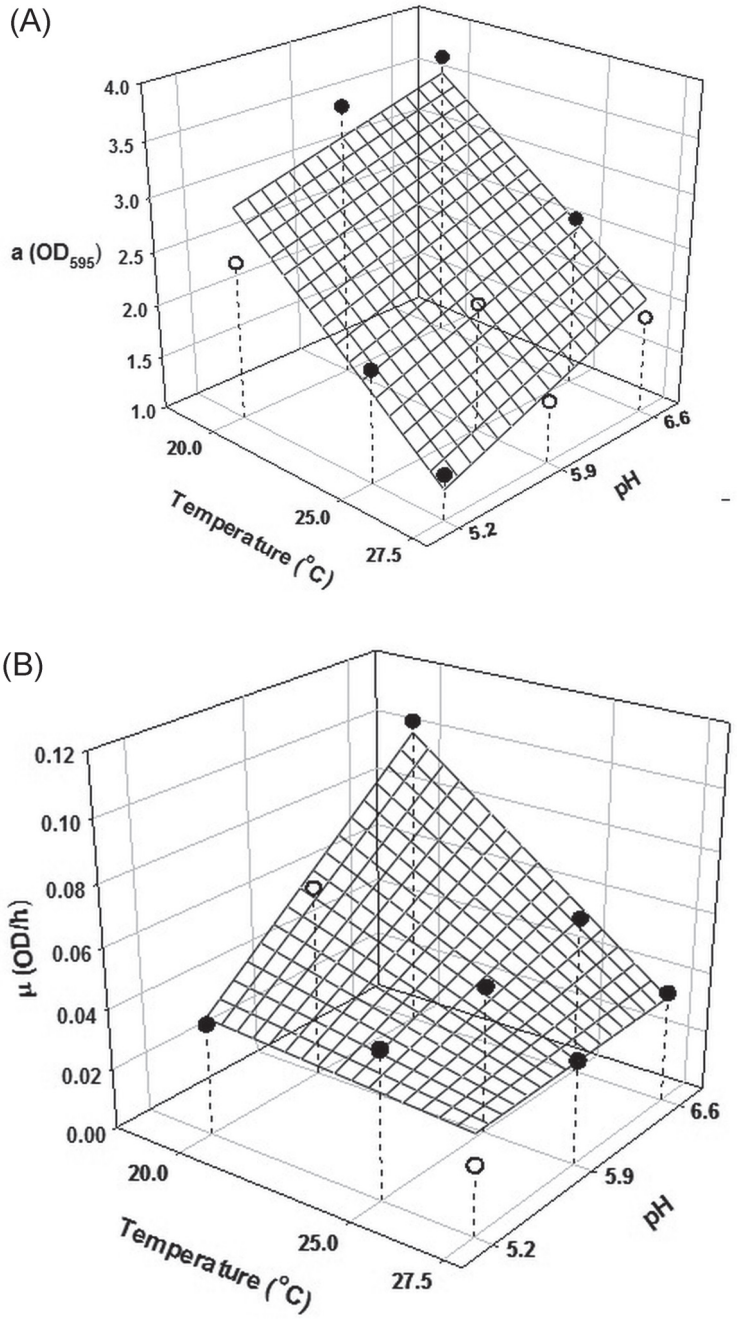
(A)
a (OD595)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
20.0
25.0
27.5
Temperature (°C)
5.2
5.9
6.6
pH
(B)
μ (OD/h)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
20.0
25.0
27.5
Temperature (°C)
5.2
5.9
6.6
pH

Fig. 3. (A), (B)

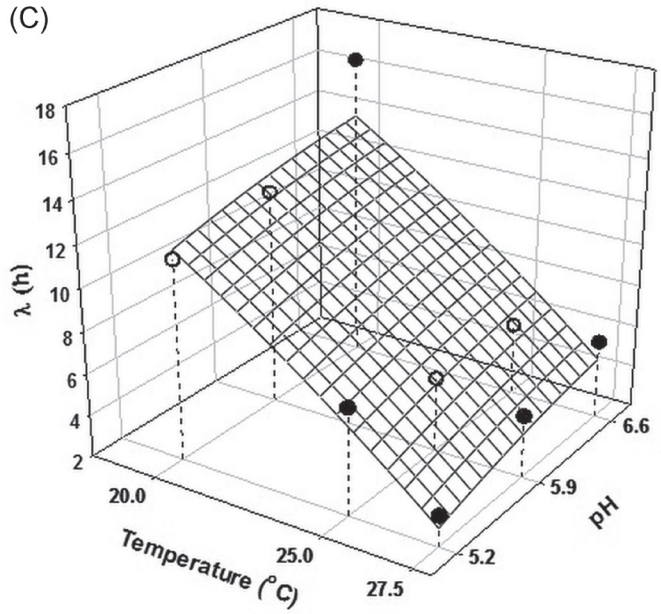

Fig. 3. Three dimensional surfaces of the quadratic polynomial model [Eq. (2) with significant coefficients] for maximum biofilm quantity (A), maximum biofilm formation rate (B) and time of acceleration of biofilm formation (C), respectively. Experimental data points: under (open circles) and above (closed circles) the surface

Table 2
Coefficients of Eq. (2) ± standard errors together with adjusted determination coefficient (R^2_{adj}) and mean square error (MSE) values of each fit

Parameter of Eq. (1)	Coefficient of Eq. (2)		R^2_{adj}	MSE
a (Maximum biofilm quantity), (OD)	c_0	7.5 ± 0.7	0.88	0.08
	c_1	–0.34 ± 0.05		
	c_2	0		
	c_3	0		
	c_4	0		
	c_5	0.02 ± 0.007		
μ_m (Maximum biofilm formation rate), ($OD \cdot h^{-1}$)	c_0	–0.76 ± 0.23	0.87	7.2×10^{-5}
	c_1	0.029 ± 0.009		
	c_2	0		
	c_3	0.16 ± 0.04		
	c_4	0		
	c_5	–0.006 ± 0.002		
λ (Time of acceleration of biofilm formation), (h)	c_0	0	0.81	3.6
	c_1	0		
	c_2	−0.025 ± 0.004		
	c_3	6.5 ± 1.4		
	c_4	−0.43 ± 0.10		
	c_5	0		

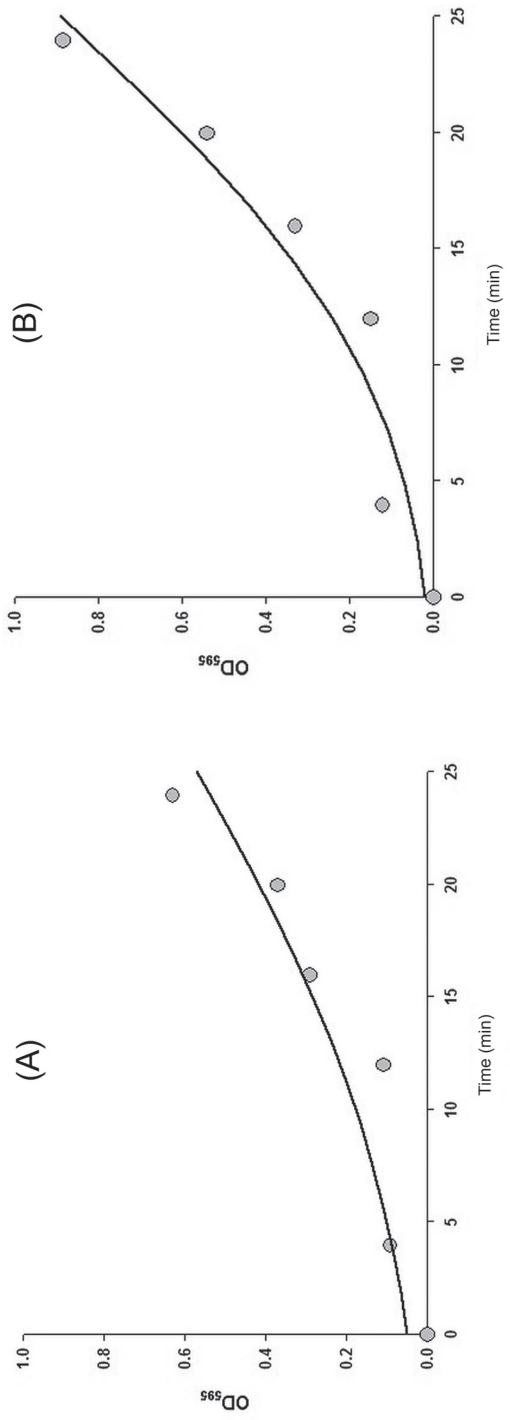

Fig. 4. Prediction of biofilm formation data of *Salmonella* Virchow up to 24 h at 23 °C, pH 5.3 (A), 23 °C, pH 6.3 (B), 26 °C, pH 5.3 (C), 26 °C, pH 6.3 (D)

time of acceleration of biofilm formation (λ) also increased (Fig. 3). Table 2 shows the coefficient values (insignificant ones were removed) and their standard errors together with R^2_{adj} and MSE values.

Predicting the biofilm formation up to 24 h

Primary [Eq. (1)] and secondary [Eq. (2)] models produced reasonable fits, therefore the predictive capability of the proposed models was further investigated: Eq. (2) used for describing the temperature and pH dependence of *a*, μ_m and λ was inserted into Eq. (1) to predict the biofilm formation of *S.* Virchow at different temperature and pH values. Two temperatures (23 and 26 °C) and pH (5.3 and 6.3) values were used up to 24 h for prediction study. Figure 4 shows the prediction of biofilm formation at two different temperatures and pH values.

DISCUSSION

Salmonella cells can form biofilms on biotic and abiotic surfaces [14, 26–29]. It is known that bacteria regulate gene expression in response to different environmental signals such as temperature, osmolarity, O_2, CO_2, pH, nitrogen compounds, nutrient availability, inorganic concentrations [9, 26, 28].

In this study we have shown that *S*. Virchow strain isolated from Turkey is able to produce biofilm on polystyrene surface. The findings is very disturbing, because plastic materials are commonly used in the food and health industries place like, food-processing plants, slaughterhouse, pipe-work, tanks, accessories in the kichen and cutting surfaces [34, 37]. According to several authors [34, 38], a large number of bacteria including *Salmonella,* adhere to hydrophobic (rubber and plastic) surfaces than to hydrophilic (glass and stainless steel) surfaces.

Nguyen et al. [21] observed that the rate of biofilm formation increased with increasing temperature (28, 37 and 42 °C) and pH (6 and 7). On the other hand, Stepanovic et al. [36] found that the quantities of biofilm formed by 30 different *Salmonella* spp. after 24 h at 30 °C were statistically higher than those formed at 37 or 22 °C. However, after 48 h of incubation the statistically highest quantity of forming biofilm was found at 22 °C. Norwood and Gilmour [22] concluded that the optimum temperature for biofilm formation by *Listeria monocytogenes* was 18 °C, and that is in agreement with the results of the Stepanovic et al. [36] for *Salmonella* spp. and current study for *S.* Virchow. The results shown for biofilm production by *Salmonella* spp. at lower temperature values (20–22 °C) emphasizes the necessity for regular and appropriate cleaning in food processing plants [36]. Chavant et al. [3] observed that temperature affects the bacterial hydrophilic surface properties, especially in low temperature and change the bacteria's ability to adhere to hydrophobic materials. Likewise, Castelijn et al. [2] showed that at 37 °C, the strains of *S.* Typhimurium (51 stains) generally showed less biofilm formation than at 25 °C.

This study also revealed that at lower temperature values higher biofilm production could be observed for *S.* Virchow (Fig. 1).

In most of the published studies, biofilm formation was quantified at a single time point [6, 19] which may be inadequate to evaluate the complete pattern of biofilm formation under different temperature and pH values [21]. In this study biofilm formation up to 96 h were monitored at three different temperature and pH values to observe the biofilm formation pattern and to describe the biofilm formation by use of a model. It should be noted that modeling study could not be conducted with various number of temperature and pH levels within a wide range. The reason of using limited number of temperature and pH levels within a narrow range is that low biofilm formations were observed at temperatures below 20 and above 27.5 °C and the same was also described for pH.

The biofilm formation of *S.* Virchow with respect to time (Fig. 1) was similar to growth curves of many bacteria therefore, a common model [Eq. (1)] that is being used for describing the growth of bacteria was preferred to model the biofilm formation. The results indicated that this model can be adequately used to describe the biofilm formation of *S.* Virchow (Table 1 shows that high R^2_{adj} and low MSE values of each fit revealing that Eq. (1) could be successfully used to describe the biofilm formation of *S.* Virchow under all tested conditions); however, similar models with three adjustable parameters could also be used with the same goodness-of-fit. Speranza et al. [32] used the same equation [Eq. (1)] to calculate aptitude to biofilm formation (λ) of *Salmonella* sp. (time necessary to start adhesion on the surface). Karaca et al. [16] also used the same equation [Eq. (1)] to describe the biofilm formation of 19 out of 25 *Salmonella* strains including *S.* Virchow at 20 °C.

It could be also possible to use different equations as the secondary model to define the temperature and pH dependency of primary model parameters; however, polynomial equation produced the best goodness-of-fit among the alternatives (data not shown). Precautions such as cleaning and disinfection of the surface could be taken to avoid the biofilm formation before λ is reached [16] because if biofilms are formed by pathogenic microorganisms in the food environment, it is very difficult for them to be completely destroyed or removed from the food-processing facilities [30]. The λ values shown in Fig. 3C were all less than 24 h, moreover it is known that *Salmonella* cells could be able to start adhesion and biofilm formation within only 6 h, therefore the predictions of biofilm formation were performed during the first 24 h. The predictions from the integrated model [Eq. (2) was inserted into Eq. (1)] were shown in Fig. 4. As mentioned before Karaca et al. [16] and Speranza et al. [32] used the same equation to describe the biofilm formation; however, to the best of our knowledge this is the first study in which prediction of biofilm formation at different temperature and pH levels was performed.

The biofilm formation is a complex phenomenon affected by several factors such as chemical and physical properties of cell surface, attachment surface and composition of surrounding medium [21]. The reasons of the differences between model predictions and experimental values may be due to oversimplification of the complex

nature of the biofilm formation with mathematical models. Nevertheless, although not perfectly matched with the experimental values, reasonable estimates were observed especially at 23 °C.

In conclusion, it has been demonstrated that modeling and predicting the biofilm formation at different temperature and pH values could be possible. The present findings could be extended for different microorganisms on different surfaces under various conditions. Moreover, modeling could be a powerful tool to take precautions to prevent biofilm formation.

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