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Prediction of *Escherichia coli* O157:H7 adhesion and potential to form biofilm under experimental conditions

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ABSTRACT

Escherichia coli O:157:H7 adhesion and potential to form biofilm on three different surfaces commonly used in the food industry was evaluated using probabilistic models; the surfaces tested were stainless steel 304 (SS304), poly(vinyl chloride) film covered with thick cloth (PVC1) and poly(vinyl chloride) film covered with thin cloth (PVC2). Using a Central Composite Rotational Design (CCRD), the effect of contact time (0 h, 7 h, 24 h, 41 h and 48 h) and temperature (12 °C, 17 °C, 28 °C, 39 °C and 44 °C) on the probability of achieving a particular adherent cell count (Log_{10} CFU cm^{-2}) was determined. By analyzing response surface plots and their corresponding contour plots and by determining quadratic equations for each surface, experimental values were shown to be significant in accordance with predicted values in all cases. The adjusted determination coefficient (R^2_{adj}) was 90.5%, 97.2% and 98.9% for SS304, PVC1 and PVC2, respectively, and the level of significance was $P \leq 0.001$. The bias factor (B_f) and accuracy factor (A_f) both approached 1.0 for the three surfaces evaluated. The model equations for predicting optimum response values were verified effectively by a validation data set for all surfaces evaluated. Therefore, an RSM provides a useful and accurate method for predicting *E. coli* O157:H7 adhesion and potential to form biofilm on SS304, PVC1 and PVC2 and could be considered to be a standard way to ensure food safety with respect to *E. coli* O157:H7 contamination through adhesion and biofilm formation.

1. Introduction

Escherichia coli O157:H7 (designated by its somatic, O, and flagellar, H, antigens) was first recognized as a human pathogen following two hemorrhagic colitis outbreaks in 1982. The first outbreak occurred in Oregon with 26 cases and 19 hospitalizations, and the second followed three months later in Michigan with 21 cases and 14 hospitalizations. Undercooked hamburgers from the same fast-food restaurant chain were identified as the vehicle, and *E. coli* O157:H7 was isolated from patients and a frozen ground beef patty. Outbreaks of *E. coli* O157:H7 infections have been primarily associated with eating undercooked ground beef, but a variety of other foods have also been implicated as vehicles (CDC, 2009). Cross-contamination of foods can occur in food-processing plants and during subsequent handling and preparation, resulting in a wide range of foods being implicated in outbreaks of *E. coli* O157:H7 infections. Recent studies have described an occurrence of *E. coli* O157:H7 infection due to milk consumption (Denny, Bhat, & Eckmann, 2008). *E. coli* serotype O157:H7 is a rare variety of *E. coli* but is a normal inhabitant of the intestines of all animals, including humans. The pathogen produces large quantities of one or more related potent toxins, called Shiga toxins, which cause severe damage to the lining of the intestine and to other target organs, such as the kidneys. The most severe outcome of Shiga toxin exposure among the general population is typically hemorrhagic colitis, a prominent symptom of which is bloody diarrhea. However, life-threatening complications sometimes occur. Some victims, particularly the very young, may develop hemolytic uremic syndrome (HUS). Overall, the Center for Disease Control and Prevention estimates that *E. coli* O157:H7 is responsible for approximately 73,500 infections, 2150 hospitalizations, and 61 deaths in the United States each year (CDC, 2009).

A biofilm can be defined as a sessile bacterial community of cells that live attached to each other and to surfaces, and its formation occurs on solid surfaces in contact with a liquid. Organic and inorganic material in the liquid can deposit as sediment on the solid surface. Subsequently, biologically active microorganisms attract to

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this conditioned surface and adhere to it. The microbial cells can then initiate growth, form an attachment matrix and develop into a complex community forming a microbial biofilm. The adhesion of bacterial cells in this manner to food-processing equipment can lead to a variety of problems, such as corrosion of metal surfaces and cross-contamination of processed foods; biofilms can also develop on food surfaces (Cooley, Miller, & Mandrell, 2003; Costerton, 1995; Gabis & Faust, 1988). While in a biofilm, the microbial community as a whole and specific individual cells exhibit increased resistance to sanitation measures and disinfectants, which makes it difficult to remove the biofilm or to inactivate particular microorganisms (Kumar & Anand, 1998). *E. coli* O157:H7 is known to produce exopolysaccharides (EPS), which can provide a physical barrier to protect cells against environmental stresses. EPS is also involved in cell adhesion and biofilm formation (Junkins & Doyle, 1992). EPS can serve as a conditioning film on inert surfaces, affect cell attachment by functioning as an adhesive or antiadhesive and influence the formation of three-dimensional biofilm structures (Ozer & Demirci, 2006; Ryu & Beuchat, 2005; Tamplin, Paoli, Marmer, & Phillips, 2005).

Predictive microbiology is a specific application of the field of mathematical modeling used to describe the behavior of pathogenic and spoilage microorganisms under a given set of environmental conditions. Growth-predictive models have been widely accepted as informative tools that can provide quick and cost-effective assessments of microbial growth for product development, risk assessment, and educational purposes. Although there are several classifications of predictive models, the classification proposed by Whiting and Buchanan (1993) (primary, secondary, and tertiary models) is currently the most commonly used.

In predictive microbiology, the development and application of secondary models for growth rate and lag time have been extensively reviewed (Devlieghere et al., 2000, 2001; McClure, Beaumont, Sutherland, & Roberts, 1997; Zurera-Cosano, Castillejo-Rodríguez, García-Gimeno, & Rincón-León, 2004). Square-root models describe the effect of suboptimal temperatures on growth rate. When this initial model is fitted to experimental growth rates, the data are square-transformed to stabilize their variance. This empirical relationship was transformed into a multiplicative model to consider the effects of additional environmental parameters such as CO_2, sodium lactate, or water activity (Dalgaard, Mejlholm, & Huss, 1997; Devlieghere et al., 2000, 2001). Some of the major advantages of these models are that they are simple, easy to interpret, and use few parameters. Furthermore, the biological significance of a microorganism's behavior can be obtained from the restricted parameters. Polynomial models were extensively used in the 1990s and are the most common secondary models. They do provide certain advantages; for example, it is easy to fit heterogeneous groups of experimental data using multiple linear regression techniques.

Response surface methodology (RSM), an empirical modeling technique used to estimate the relationship between a set of controllable experimental factors and observed results, is currently one of the most popular optimization techniques in the field of food science because of its comprehensive theory, reasonably high efficiency, and simplicity. The number of experimental points in the Central Composite Rotational Design (CCRD) is sufficient to test the statistical validity of the fitted model and the lack-of-fit of the model. Among secondary models, RSM has been commonly used in predictive microbiology to describe the effects of environmental dependences on the growth parameters of microorganisms. Similar to previous reports, RSM have been shown to successfully as a function of factors, such as pH, NaCl, temperature, and other preservatives (Buchanan & Phillips, 1993; McClure et al., 1997; Wijtzes, Rombouts, Kant-Muermans, Van't Riet, & Zwietering, 1993) for the growth of *Clostridium sporogenes* (Dong, Tu, Guo, Li, & Zhao, 2007) and *Leuconostoc mesenteroides* (Zurera-Cosano, García-Gimeno, Rodríguez-Pérez, & Hervás- Martínez, 2006), death of *Salmonella* Enteritidis (Koutsoumanis, Lambropoulou, & Nychas, 1999), growth rate and lag time of *Listeria monocytogenes* (Augustin & Carlier, 2000; Carrasco et al., 2006) and inactivation of *Listeria monocytogenes* (Gao, Ju, & Jiang, 2006) under different experimental conditions.

The objectives of this study were to describe *E. coli* O157:H7 adhesion and potential to form biofilm on stainless steel AISI 304, poly(vinyl chloride) film covered with thick cloth and poly(vinyl chloride) film covered with thin cloth at 12–44 °C for different contact times, and to develop and validate RSM using CCRD for mathematically predicting *E. coli* O157:H7 adhesion on these different surfaces commonly used by food industries.

2. Materials and methods

2.1. Bacterial strains and culture conditions

An aliquot of stock culture containing *E. coli* O157:H7 (strain ATCC 43895) kept at −25 °C in 15% (w/v) glycerol was inoculated in 10 mL of Nutrient broth (NB) and incubated for 48 h at 37 °C. Then, 10 mL of the culture was subcultured in 90 mL of NB, and the procedure was repeated for three consecutive days. A third subculture was grown for 18 h in 100 mL of NB until the early stationary phase was reached. All necessary dilutions were made in NB to obtain an inoculum size of 10^4 CFU mL^{-1}.

2.2. Surfaces used and chip preparation

Stainless steel AISI 304 #4 (SS304), poly(vinyl chloride) film covered with thick cloth (PVC1) and poly(vinyl chloride) film covered with thin cloth (PVC2) were selected as surfaces of study and used to prepare the chips. The chips were cut to dimensions of 10 × 10 × 1 mm.

All chips were cleaned with a neutral detergent solution for 20 min in an ultrasonic water bath (model 250D; VWR, West Chester, Pa.) and rinsed five times in 10 mL of sterile distilled water with agitation using a vortex. Cleaned chips were sterilized by UV exposure (100 mW cm^{-2}) for 60 min. In a subsequent step, the chips were degreased in ethylic alcohol for 1 h and rinsed twice with distilled water using a vortex. To finish, they were dried in a laminar air flow cabinet (Parizzi, Andrade, Silva, Soares, & Silva, 2004).

2.3. In vitro chip adherence assay

A cleaned and degreased chip was deposited in a glass flask containing the third subculture of *E. coli* O157:H7 adjusted to a concentration of 10^4 CFU mL^{-1} in NB, and then maintained at room temperature (25 °C). A chip was removed from the glass flask using sterile tongs and rinsed in 10 mL of 0.1% peptone water for 1 min to remove planktonic cells. Following this, the chip was transferred to a tube containing 5 mL of 0.1% peptone water and rinsed for 1 min with shaking using a vortex (model QL-901, Biomixer®, maxima potency: 2800 rpm) to remove attached cells (Parizzi et al., 2004). The cell adhesion to each surface was analyzed after 0 h, 2 h, 4 h, 6 h, 8 h and 10 h of contact.

The number of bacteria that adhered to each chip was evaluated using the plate count method. The number of CFU recovered from each chip was determined by plating appropriate serial dilutions in 0.1% peptone water on MacConkey agar plates and incubating them at 37 °C for 48 h. Each colony counted on a plate was equivalent to 2.5×10^2 CFU cm^{-2}, which was obtained by multiplying the diluent volume (5 mL) by the plated aliquot (1 mL) and dividing by the total surface area of the chip (2 cm^2). Time zero (0 h) corresponds to

analyses conducted after chip had been immersed in and immediately removed from a glass flask containing NB and bacterial suspension.

All experiments were replicated three times; in each experiment, two chips were examined per replicate.

2.4. Experimental design for the evaluation of cell adhesion using RSM

This investigation involved the use of CCRD, which was arranged with two independent variables used to obtain the combination of values that optimizes response within the region of a three-dimensional observation space and four axial points to allow the design a minimal number of experimental runs according to Montgomery (1991), pp. 39–76. Two different parameters (i.e., temperature and contact time) were chosen as key variables and designated as T and t, respectively. The low, middle, and high levels of each variable were designated as $-\sqrt{2}$, 0, and $+\sqrt{2}$, respectively, and are given in Table 1. The variables were coded according to the following equation:

$$b_i = B_i - \frac{B_0}{\Delta B}$$

where b_i is the (dimensionless) coded value of the variable B_i; B_0 is the value of B_i at the center point, and ΔB is the step change. The behavior of the system was explained by the following second-degree polynomial equation:

$$Y = b_0 + \sum_{i=1}^{2} b_1 T + \sum_{i=1}^{2} b_2 T^2 + \sum_{i=1}^{2} b_3 t + \sum_{i=1}^{2} b_4 t^2 + \sum_{i=1}^{2} b_5 T \cdot t$$

where Y is the predicted response (adherent cell count (CFU cm^{-2} of *E. coli* O157:H7)), T and t are input variables (temperature (°C) and contact time (h) respectively); b_0 is a constant; b_1 and b_3 are linear coefficients; B_5 is a cross-product coefficient; and b_2 and b_5 are quadratic coefficients.

The central point in CCRD was replicated several times to estimate variance due to experimental or random variability. All tests were performed in a randomized order to exclude the effects of environmental conditions.

Cell adherence was evaluated at 12 °C, 17 °C, 28 °C, 39 °C and 44 °C and after 0 h, 7 h, 24 h, 41 h and 48 h of each contact time (Table 2), and the results were expressed as Log_{10} values for both observed and predicted counts.

A total of 12 runs were necessary to determine the optimal levels of the range of selected factors.

2.5. Internal validation of models

After establishing the parameters of the RSM, a new temperature value and two contact times in the interpolation region were randomly selected for validation of the model. For this purpose, biofilm formation and the number of adherent cells were evaluated after 22 h and 39 h of contact time, respectively, at 35 °C.

Table 1
Adhered cell count (Log_{10} CFU cm^{-2}) on the three surfaces (SS304, PVC1 and PVC2) after different contact times by the plate count method.

Surfaces	Contact time (h)					Equations	R^2
	2	4	6	8	10		
SS304	0.96^{aA}	2.26^{bA}	3.55^{cA}	4.26^{dA}	4.78^{eA}	$Y = 0{,}972x - 0.692$	0,96
PVC1	2.03^{aB}	3.81^{bB}	5.68^{cB}	6.06^{dB}	6.67^{dB}	$Y = 1{,}165x + 0.098$	0,97
PVC2	1.99^{aB}	3.75^{bB}	5.62^{cB}	6.39^{dB}	7.04^{dB}	$Y = 1{,}249x - 0.156$	0,98

Averages followed by the same lowercase letter in line (contact time) and means followed by same capital letter in column (surface) did not differ according to the Scott–Knott test ($P \geq 0.05$). SS304 = stainless steel; PVC1 = polyvinyl chloride coated with thick cloth; PVC2 = poly(vinyl chloride) film covered with thin cloth. Y = Adhered cells count (Log_{10} CFU cm^{-2}), x = contact time (h).

Table 2
Factors and levels from central composite rotational design.

Factors		Level				
Variable	Code	$-\sqrt{2}$	-1	0	1	$\sqrt{2}$
Temperature	(T, °C)	12	17	28	39	44
Contact time	(t, h)	0	7	24	41	48

No standard method or set of criteria has been published by which a model should be evaluated; therefore, in this paper, the accuracy of RS models was evaluated by determining four values: root-mean-squares error (RMSE), standard error of prediction (% SEP), bias factor (B_f) and accuracy factor (A_f) (Garcia-Gimeno, Barco, Rinco, & Zurera-Cosano, 2005; Ross, 1999; Zurera-Cosano et al., 2006), which are shown as follows:

$$\text{RMSE} = \sqrt{\sum \frac{(obs - pred)^2}{n}} \quad \%\text{SEP} = \frac{100}{mean\ obs}\sqrt{\sum \frac{(observed - predict)^2}{n}}$$

$$B_f = 10^{\left(\frac{\sum \log \frac{pred}{obs}}{n}\right)} \quad A_f = 10^{\left(\frac{\sum \left|\log\left(\frac{pred}{obser}\right)\right|}{n}\right)}$$

where *pred* = the number of adherent cells predicted from the model; *obs* = the number of adherent cells observed experimentally; *mean obs* = the mean of all *obs* values; and n = the number of observations.

2.6. Statistical analysis

Data from the *in vitro* chip adherence assay at 37 °C, using the plate count method (PCM) to count adherent cells, were analyzed as a split plot design consisting of two variables: surfaces and contact time. The average cell adhesion counts (CFU cm^{-2}) on the surfaces were compared using the Scott–Knott test. The results were analyzed by an ANOVA. The coefficients of linear regressions for contact time were analyzed using a T test at 5% probability for each surface.

"MINITAB^{R}" 13.3 (Minitab Inc., USA) was used for regression analysis of the data obtained and for estimation of the coefficients of the regression equation obtained by MSR. Significant differences in mean values are presented at a 95% confidence level ($P < 0.05$). The two-dimensional graphical representation of the system's behavior, called the response surface, was used to describe the individual and cumulative effects of the variables, as well as the mutual interactions between the dependent variables and independent variables (Yu-Long, Xing-Rong, & Han-Hu, 2006).

3. Results and discussion

3.1. In vitro adherence to chips at 37 °C

Table 1 summarizes the counts of adherent cells of *E. coli* O157:H7 determined by the PCM for all surfaces evaluated at 37 °C. Data not shown indicated that the parameters used to remove cells attached to surfaces were efficient and that increasing the time or intensity of agitation did not increase the release of cells from surfaces under the conditions studied. It can be observed that the number of attached cells differed ($P < 0.05$) depending on the type

of surface among equal time points. There was a similar cell-adhesion pattern between surfaces during the evaluation time, as can be observed in Fig. 1. The number of microorganisms that are adherent to each surface increases with increasing contact time because the probability of collisions between the cell and the surface is time dependent, as seen in Table 2. Similar numbers of adherent cells were observed between the PVC2 and PVC1 surfaces but not the SS304 surface. Adhesion was always time dependent. It was also possible to observe that the mass of adherent cells was always smaller on the SS304 surface. These results confirm that the adhesion and subsequent biomass production of the biofilm are time dependent and agree with studies conducted by Notermans, Dormans, and Mead (1991) and Marshall (1992). Also, Czechowski (1990) found that bacteria adhere almost immediately to surfaces that they come in contact with, even at low temperatures such as 5 °C and 11 °C. However, the consolidation and subsequent increase in population of the biofilm are dependent on time, and the growth of some microorganisms may be attenuated by low temperatures (Pompermayer & Gaylarde, 2000).

In the food industry, the cleaning and sanitizing of solid surfaces is usually performed every 8 h. The results from this study show that an 8 h time interval is adequate for cells to attach and form biofilm on stainless steel and PVC surfaces; this represents a problem for the food industry.

Previous studies have shown that *E. coli* O157:H7 can form biofilm on stainless steel surfaces (Dewanti & Wong, 1995; Ryu, Kim, & Beuchat, 2004, Ryu, Kim, Frank, & Beuchat, 2004) and that cell detachment can result in cross-contamination during food processing. However, there are few studies focusing on the *E. coli* adhesion process to surfaces such as PVC, and most of them refer to studies in the medical field where the pathogen was isolated from tools used for health services.

A mature biofilm is usually established within 24–48 h and can contain up to 10^{10} cells cm^{-3} and 20–30 layers of bacteria. Biofilm formation is a slow process, and depending on culture conditions, it can take anywhere from a few hours to several days or months to reach a state of equilibrium (Marshall, 1992). At each time interval evaluated in this study, it was possible to observe a significant difference between the numbers of cells that had adhered to the surfaces tested. A total of 10^3 adherent cells were observed after 6 h of contact time with SS304, and the total number of adherent cells increased up to 10^4 after longer contact time. For the other two surfaces, 10^3 adhered cells was observed after approximately 3 h of contact time. There are controversies concerning the quantity of adherent cells that are necessary to form a biofilm. Ronner and Wong (1993) claim that 10^3 adherent cells cm^{-2} are necessary for biofilm formation to occur. However, Wirtanen, Husmark, and Mattila-Sandholm (1996) and Andrade, Bridgeman, and Zottola (1998) demonstrated that roughly 10^5 and 10^7 adherent cells cm^{-2}, respectively, are needed for biofilm formation to occur. Considering the very strict control to prevent biofilm formation in this study, with reference to the smaller amount of attached cells needed to form it, it is possible to observe that on the PVC1 and PVC2 surfaces, biofilms can begin to form before 3 h of contact time; on SS304, they can form before 6 h.

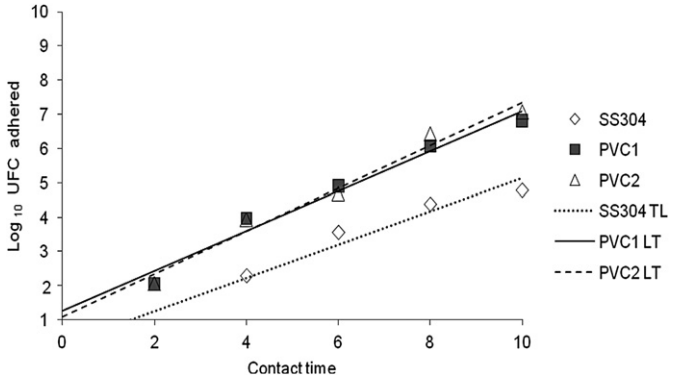


Fig. 1. Pattern of adhesion in the stainless steel (SS304) and poly(vinyl chloride) film covered (PVC 1 and PVC2) at 37 °C and respectives tendencies lines.

These results increase the urgency to develop and study predictive models that can evaluate *E. coli* O157:H7 adhesion and biofilm formation under different conditions to help the food industry keep their products safe and to prevent *E. coli* adhesion to various surfaces.

3.2. Response surface model (RSM)

Table 3 shows the CCRD arrangement for different surfaces and summarizes the response values (adherent cell count) obtained under different experimental conditions for each surface evaluated (SS304, PVC1, and PVC2).

The RSMs were elaborated by applying multiple regression analysis to the experimental data to produce the best fit and prediction accuracy. The results show a second-order polynomial equation for each surface evaluated (Eqs. (1), (2), (3)).

The terms T (temperature), t (contact time), and their interactions and quadratic terms had significant effects on the model for the PVC1 and PVC2 surfaces; therefore, they were kept in the equation. For the SS304 surface, only the terms that had a significant effect on the model remained in the equation. The coefficient values of polynomials were tested for their significance and are listed in Table 4.

$$y = -6.16110 + 0.62496T - 0.00830T^2 + 0.09510t + 0.0094t^2, \quad (1 - \text{SS304})$$

$$y = -5.56777 + 0.57067T - 0.00700T^2 + 0.21842t - 0.00114t^2 - 0.00382Tt, \quad (2 - \text{PVC1})$$

$$y = -5.38696 + 0.59426T - 0.00764T^2 + 0.17149t - 0.00100t^2 - 0.00262Tt. \quad (3 - \text{PVC2})$$

These data corroborate those observed under previously evaluated optimal conditions (37 °C) and confirm that adhesion is always a function of contact time and temperature. Moreover, all linear coefficients are positive, which means that when the factor level increases, there is more adhesion on all surfaces evaluated. The statistical model was checked by an F value, and the ANOVA for the response surface quadratic model is summarized in Table 5.

The coefficient values of all models were calculated and tested for their significance. The model was significant for all surfaces evaluated. In Table 5, it can be seen that the model adopted for PVC1 is highly significant (F value of 206.56), followed by PVC2 (F value of 76.93) and SS304 (F value of 22.4). For all models, there is a chance of less than 0.1% that a model's F values occur randomly. Values of "$P > F < 0.05$" indicate that a model's terms are significant. Other evidence can be provided by the lack-of-fit F-values.

The error term can be partitioned into two parts: pure error (error within replicates) and lack-of-fit error, which represents degrees of freedom that are not in the model (e.g., higher-order interaction terms). An F-value is used in the lack-of-fit test to determine if the model selected is an appropriate model. If the P-value is less than the selected α-level, there is evidence of lack-of-fit.

The lack-of-fit F-values of 4.27, 5.25 and 2.14 (SS304, PVC1 and PVC2, respectively) imply that the lack-of-fit in these models is not

Table 3
CCRD arrangement for different surfaces and respective average of responses observed and predicted (Count of adhered cell).

Runs	X_1	X_2	Temperature (T, °C)	Contact time (t, h)	Adhered cells ($\log_{10}$ CFU cm^{-2})					
					SS304		PVC1		PVC2	
					Obs[a]	*Pred*[b]	*Obs*[a]	*Pred*[b]	*Obs*[a]	*Pred*[b]
1	$-\sqrt{2}$	0	12	24	1.706	2.067	3.549	3.759	3.339	3.426
2	−1	−1	17	7	2.563	2.406	3.230	3.130	3.379	3.346
3	−1	1	17	41	5.900	5.366	6.778	6.490	6.163	6.022
4	0	0	28[c]	24	5.414	5.558	6.718	6.945	6.894	7.037
5	0	0	28[c]	24	5.285	5.558	7.082	6.945	6.986	7.037
6	0	0	28[c]	24	5.264	5.558	7.028	6.945	7.009	7.037
7	0	$\sqrt{2}$	28	48	6.830	7.364	7.386	7.652	7.503	7.655
8	0	0	28[c]	24	5.940	5.558	7.023	6.945	7.050	7.037
9	0	0	28[c]	24	5.855	5.558	6.861	6.945	7.237	7.037
10	1	−1	39	7	5.451	5.454	6.454	6.477	6.613	6.603
11	1	1	39	41	5.452	6.078	7.145	6.980	7.436	7.318
12	$\sqrt{2}$	0	44	24	4.664	4.802	6.507	6.549	6.680	6.736

[a] Observed.
[b] Predicted.
[c] Center point conditions.

significant relative to the pure error. The probability of lack-of-fit occurring randomly is not the same for all models, varying from 7.6% for PVC1 to 10.20% for SS304 and 23.33% for PVC2. Non-significant lack-of-fit is good, and in this case, all of the models show non-significant coefficients, as can be seen in Table 5.

The quality of the models can be checked by the determination coefficient R^2 and the R^2_{adj} (multiple correlation coefficient R). The value of R^2_{adj} (>0.905) for the SS304 model suggests that the total variation of over 90.50% for the adherent cell count is attributed to independent variables and that less than 9.95% of the total variation cannot be explained by the model. Values of 0.972/2.8% and 0.989/1.1% are applicable to PVC1 and PVC2, respectively. The closer the values of R^2_{adj} are to 1, the better the correlation between the experimental and predicted values (Pujari & Chandra, 2000; Wang & Lu, 2005). It is important to check the adequacy of the fitted model, because an incorrect or under-specified model can result in misleading conclusions.

The models also show statistically insignificant lack-of-fit, as is evident from the calculated F values (4.27, 5.25, and 2.14 to SS304, PVC1, PVC2 respectively), which are lower than the tabulated F value ($F_{0.05(5,2)} = 19.3$), even at the 0.05 level. The models are found to be adequate for predictions within the range of the variables employed.

The graphical representations of the regression equations, known as the response surfaces and contour plots, were obtained. Use of these graphics makes it simple and convenient to understand the interactions between temperatures and times and also to locate their optimum levels, as seen in Figs. 1, 2 and 3. According to Muralidhar, Chirurnamila, and Marchant (2001), the shape of the contour plots (circular or elliptical) indicates whether the mutual interactions between variables are significant or not. As seen in Figs. 1, 2, and 3, all contour plots are elliptical in shape, demonstrating that the models are significant; this is most evident for PVC2, followed by PVC1 and then SS304. Behavior can be confirmed by ANOVA for models of all surfaces.

Table 4
Estimated regression coefficients for adhesion cells of *E. coli* O157:H7.

Term	Coefficient	SE	t	P-value
SS304 (Eq. (1))				
Constant	−6.60248	2.26280	−2.918	0.00
T	0.43808	0.13796	3.176	0.004
t	0.28796	0.07239	3.978	0.000
$T*T$	−0.00496	0.00230	−2.156	0.040
$t*t$	−0.00214	0.00101	−2.116	0.044
$T*t$	−0.00312	0.00185	−1.691	0.102**
$R^2 = 94.8\%$ $R^2_{adj} = 90.5\%$				
PVC1 (Eq. (2))				
Constant	−5.56777	0.724697	−7.683	0.000
T	0.57067	0.043814	13.025	0.000
t	0.21842	0.028220	7.740	0.000
$T*T$	−0.00700	0.000723	−9.676	0.000
$t*t$	−0.00114	0.000394	−2.890	0.028
$T*t$	−0.00382	0.000625	−6.112	0.001
$R^2 = 98.5\%$ $R^2_{adj} = 97.2\%$				
PVC2 (Eq. (3))				
Constant	−5.38696	0.461724	−11.667	0.000
T	0.59423	0.027915	21.287	0.000
t	0.17149	0.017980	9.358	0.000
$T*T$	−0.00764	0.000461	−16.582	0.000
$t*t$	−0.00100	0.000251	−3.999	0.007
$T*t$	−0.00262	0.000398	−6.587	0.001
$R^2 = 90.4\%$ $R^2_{adj} = 98.9\%$				

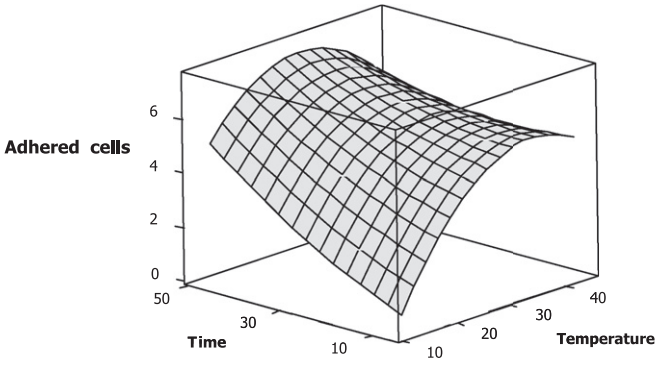
Table 5
ANOVA to fitted model for different surfaces (SS304, PVC1 and PVC2).

Source	df	SS	Adj MS	A	F	P
SS304						
Regression	5	23.8696	23.8696	4.7739	22.04	0.001
Linear	2	15.1268	11.1872	5.5936	25.83	0.001
Quadratic	2	7.3792	7.3792	3.6896	17.03	0.003
Interaction	1	1.3636	1.3636	1.3636	6.30	0.046
Residual error	6	1.2996	1.2996	0.2166		
Lack-of-fit	2	0.8849	0.8849	0.4425	4.27	0.102
Pure error	4	0.4146	0.4146	0.1037		
Total	11	25.1692				
PVC1						
Regression	5	21.0035	21.0035	4.20071	76.93	0.000
Linear	2	5.7119	11.1051	5.55254	52.30	0.000
Quadratic	2	13.2514	5.7119	2.85597	101.69	0.000
Interaction	1	2.0402	2.0402	2.04016	37.36	0.001
Residual error	6	0.3276	0.3276	0.05460		
Lack-of-fit	2	0.2373	0.2373	0.11863	5.25	0.076
Pure error	4	0.0904	0.0904	0.02259		
Total	11	21.33120				
PVC2						
Regression	5	22.8926	22.8926	4.57852	206.56	0.000
Linear	2	15.3408	10.9356	5.46778	246.68	0.000
Quadratic	2	6.5902	6.5902	3.29512	148.66	0.000
Interaction	1	0.9616	0.9616	0.96159	43.38	0.001
Residual error	6	0.1330	0.1330	0.02217		
Lack-of-fit	2	0.0688	0.0688	0.03440	2.14	0.233
Pure error	4	0.0642	0.0642	0.01605		
Total	11	23.0256				

3.3. Performance of the models

Predictive models in microbiology should be systematically validated using new data to test the robustness of the models in conditions different than those initially tested. Table 6 shows the results for the parameters used to determine the performance of the models. For all surfaces evaluated, the polynomial equations produced a low value for the RMSE statistic, which indicates a good fit of the experimental data for the model. RMSE provides a measure of the goodness-of-fit of a model to the data used to produce it. The results obtained here were more adjusted values than those observed in previous studies (Dong et al., 2007; Garcia-Gimeno et al., 2005; Zurera-Cosano et al., 2004, 2006). The standard error of prediction, % SEP, produced low values for each of the three surfaces: 6.6% for SS304, 2.4% for PVC1 and 1.5% for PVC2, thus confirming the concordance between the observed and predicted values.

Because the value B_f does not provide any indication of the average accuracy of estimates then A_f was also calculated. The larger the value of the accuracy factor (A_f), the less accurate the average estimate is. Thus, an accuracy factor of 2 indicates that the prediction is, on average, a factor of 2 away from the observed value, which means it is either half as large or twice as large; a value of 1 indicates that there is perfect agreement between all predicted and observed values. The average estimate tends to be less accurate with the increase of A_f (Lebert, Robles-Olvera, & Lebert, 2000).



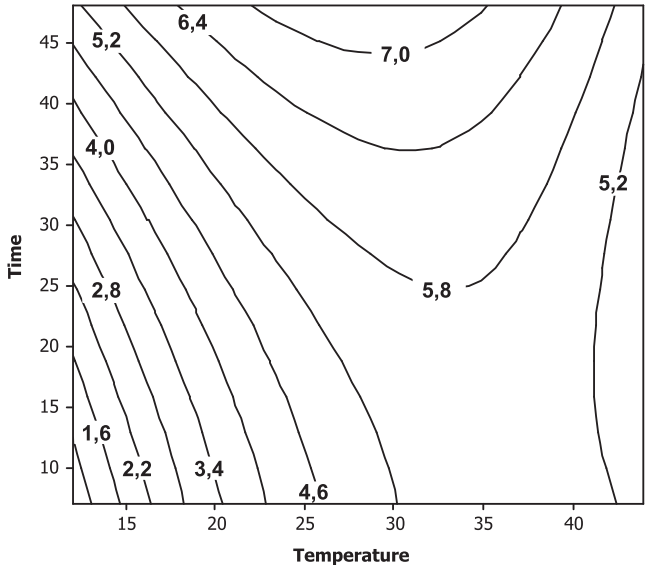
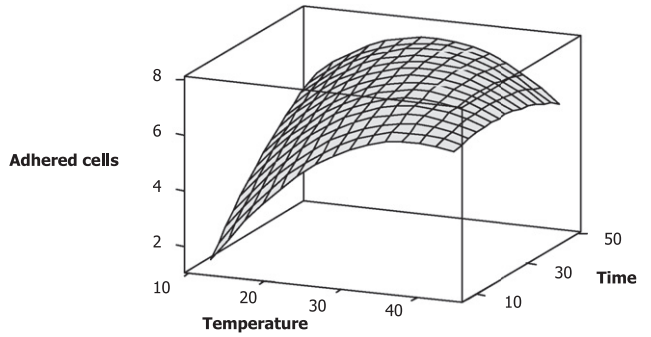


Fig. 2. Response surface plot (upper) and its corresponding contour plot of adhered cell count of *Escherichia coli* O157:H7 in SS304 as function of temperature and time.



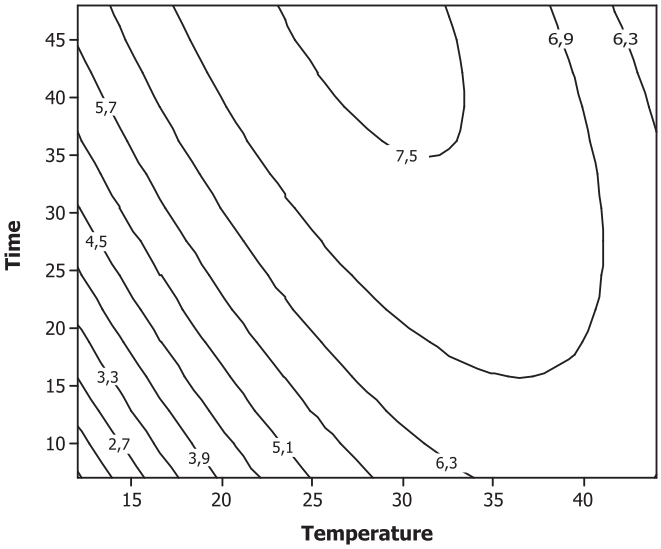


Fig. 3. Response surface plot (upper) and its corresponding contour plot of adhered cell count of *Escherichia coli* o157:h7 in pvc1 as function of temperature and time.

Ideally, predictive models should show a relationship of $B_f = A_f = 1$ (accurate and not biased). Ross (1999) stated that growth models of pathogenic microorganisms should present values for B_f of between 0.9 and 1.15. Furthermore, Ross, Baranyi, and McMeekin (2000) considered an A_f value of up to 0.15 (15%) for each factor included in the model to be acceptable. Therefore, when using 2 factors (temperature and contact time), A_f values up to 1.30 should be expected.

Assuming the above conditions in this study, as all the values of B_f and A_f were very close to 1, it is evident that the models provide adequate descriptions of the data set of observed and predicted values obtained from developed models.

In addition, a graphical comparison was performed to illustrate the quality of the proposed RS model by plotting predicted against observed adherent cell counts for each surface (Fig. 4 a, b, and c). The points for observed and predicted values are evenly distributed around the diagonal, and the points are close to the line of 100% agreement, indicating an excellent model to estimate *E. coli* O157:H7 adhesion for all surfaces evaluated (data not shown).

Table 6
Mathematical external evaluation based on adhered cell count by RS models for model validation on three different surfaces (SS304, PVC1 and PVC2).

Surfaces	RMSE	%SEP	B_f	A_f
SS304	0.360	6.62	1.0083	1.0720
PVC1	0.165	2.42	1.0007	1.0266
PVC2	0.105	1.52	0.9950	1.0235

RMSE = root-mean-squares error; %SEP = standard error of prediction; B_f: Bias factor; A_f: accuracy factor.

Although the equations used in the models were empirical, they were shown to be satisfactory.

3.4. Validation of the models

As stated in the Section 2, the models were validated using additional data collected under different experimental conditions from those included in the models (Table 7). From Table 7, it can be seen that for all surfaces, the predicted values were above the observed values. As a consequence, it can be stated that the prediction is fail-safe using these models. This is not surprising because the models were quite satisfactory in predicting *E. coli* adhesion, as confirmed by F-test, RMSE, % SEP, bias factor (B_f) and accuracy factor (A_f). As prediction conditions were more toward the center of the ranges covered by the experimental design, the results were expected to be most precise. Some previous authors have also attempted to validate models of foods contamination and to discuss the sources and types of error associated with this validation (Baranyi, Pin, & Ross, 1999; Neumeyer, Ross, Thomson, & McMeekin, 1997; Ross, 1999). It is important to continue to improve the accuracy and representational value of the models so that they will give realistic predictions without being excessively fail-safe. This will promote confidence in the value of predictive models within the food industry and encourage their application in real situations, with consequent economic benefits. However, good results cannot be obtained if the empirical models are used to make predictions outside the experimental ranges that the models

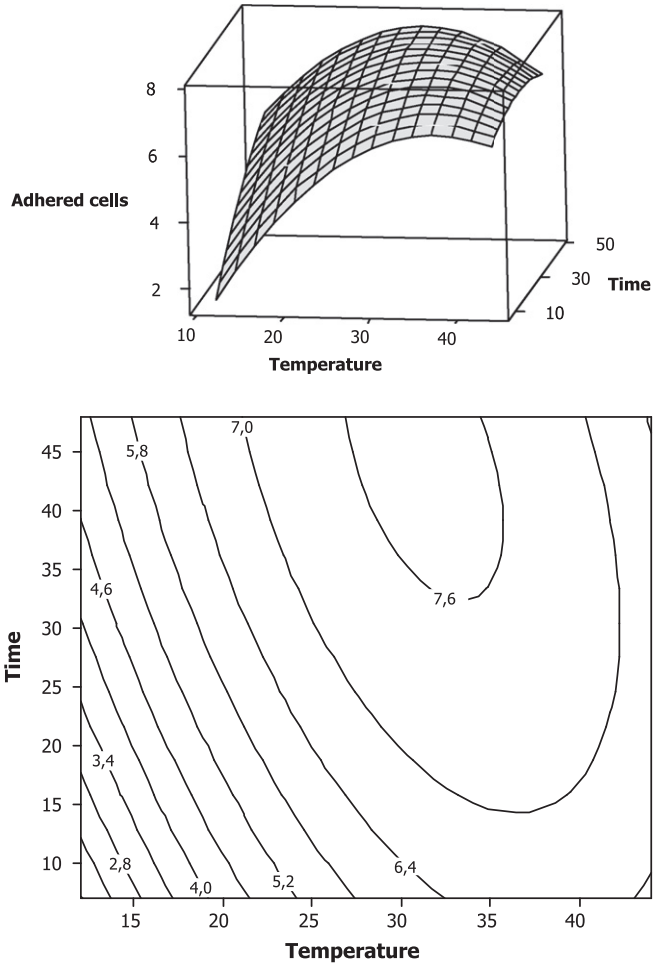


Fig. 4. Response surface plot (upper) and its corresponding contour plot of adhered cell count of *Escherichia coli* O157:H7 in PVC2 as function of temperature and time.

Table 7
Response of experimental and surface model as a function of temperature and contact time for additional conditions randomly selected from mathematical validation of model.

Temperature (°C)	Contact time (h)	SS304		PVC1		PVC2	
		Obs[a]	*Pred*[b]	*Obs*[a]	*Pred*[b]	*Obs*[a]	*Pred*[b]
35	22	5.32	5.66	6.25	7.26	7.30	7.34
35	39	6.20	6.53	6.79	7.72	7.54	7.87

[a] Observed.
[b] Predicted.

were created to test. Because empirical models are based on a statistically determined fit of the data, in conditions approaching the limits of the model, it becomes less likely that extrapolations will be accurate. Baranyi et al. (1999) and Pin, Sutherland, and Baranyi (1999) emphasized the importance of the correct definition of the interpolation region of a model. The selection of the correct interpolation region (and not just a random range of variables) is essential to completes description of the empirical model. The intervals of temperature and contact time studied were selected to fulfill answer this facet of the study.

All results from this study confirm that predictive microbiology is an important tool in the quality control area of the food industry to predict the behavior of microorganisms, and its main objective is the use of mathematical models to describe the behaviors of complex systems such as food-microorganisms under the influence of intrinsic environmental factors and extrinsic factors. In our case, these factors were temperature and contact time with three different surfaces commonly used in the food industry.

Over the years, various studies have contributed to a better understanding of microbial behavior during biofilm formation. Some studies revealed remarkable variation in the quantity of cells needed to begin biofilm formation (Andrade et al., 1998; Dewanti & Wong, 1995; Wirtanen et al., 1996), the role of EPS production (Ryu, Kim, & Beuchat, 2004; Ryu, Kim, Frank et al., 2004), and the variability between different *E. coli* isolates to form biofilms (Reisner, Krogfelt, Klein, Zechner, & Molin, 2006) *in vitro* under a variety of growth conditions. The importance of environmental conditions emphasizes the need to develop better biofilm model systems that will approximate *in vivo* situations by adjusting for the medium composition, diversity of the microbial community present, initial number of cells, and different surfaces, among other variables.

4. Conclusions

This work is important because it extends the knowledge of potential *E. coli* O157:H7 adhesion and biofilm formation on surfaces commonly used in the food industry. Cross-contamination of foods is one of the major concerns in the food industry, and if microorganisms are not completely removed from food-contact surfaces, they may go on to form biofilms and also increase the bio-transfer potential. The models' successes in predicting optimum response values were verified effectively using a validation data set for all three surfaces evaluated. For that reason, RSM provide useful and accurate methods for predicting *E. coli* O157:H7 adhesion on SS304, PVC1 and PVC2. Although extensive mathematical evaluation proved their adequacy, caution should still be taken when putting the models into practice. They need validation in a food industry environment before being applied in practice, and before they can be considered as a standard by which to ensure food safety in respect to *E. coli* O157:H7 contamination due to adhesion.

There are still many unknown factors concerning the adhesion capacities of major food pathogens. The parameters that govern the adhesion of bacteria into solid surfaces are not yet all understood, and the study of these parameters could help develop new

prevention procedures at the initial stages of microbial adsorption. However, in subsequent studies, it is important to consider the development of models that will enable the simultaneous evaluation of the multiple species present in biofilms and their possible antagonistic or synergistic interactions.

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