Food Microbiology 83 (2019) 95–103

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

An ordinal logistic regression approach to predict the variability on biofilm formation stages by five *Salmonella enterica* strains on polypropylene and glass surfaces as affected by pH, temperature and NaCl

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ARTICLE INFO

Keywords:
Attachment
Cleaning
Disinfection
Food safety
Cross contamination
Predictive microbiology

ABSTRACT

This study assessed the adhesion and formation of biofilm by five *Salmonella enterica* strains (*S.* Enteritidis 132, *S.* Infantis 176, *S.* Typhimurium 177, *S.* Heidelberg 281 and *S.* Corvallis 297) on polypropylene (PP) and glass (G) surfaces as affected by pH (4–7), NaCl concentration (0–10% w/v) and temperature (8–35 °C). Sessile counts < 3 log CFU/cm^2 were considered lack of adhesion (category 1), while counts ≥ 3 and < 5 log CFU/cm^2 corresponded to adhesion (category 2) and counts ≥ 5 log CFU/cm^2 corresponded biofilm formation (category 3). The obtained results categorized in these three responses were used to develop ordinal regression models to predict the probability of biofilm stages on PP- and G-surfaces. The experimental outcomes for lack of adhesion were > 90% on PP- and G-surfaces. Generally, adhesion outcomes corresponded to approximately 36% of the total, whereas biofilm outcomes were close to 65% in both PP- and G-surfaces. The biofilm stages varied among the strains studied and with the material surface under the same experimental conditions. According to the generated ordinal models, the probability of adhesion and biofilm formation on PP-surface by the five *S. enterica* strains tested decreased at pH 4 or 5 in NaCl concentrations > 4% and at a temperature < 20 °C. On G-surface, the probability of adhesion increased pH 6 or 7, in the absence of NaCl and temperatures < 20 °C, while, the probability of biofilm formation increased in the same pH, NaCl concentration up to 4% and temperatures ≥ 20 °C. This is the first study assessing the biofilm formation through categorical, ordinal responses and it shows that ordinal regression models can be useful to predict biofilm stages of *S. enterica* as a function of pH, NaCl, and temperature or their interactions.

1. Introduction

Salmonella enterica causes approximately 93.8 million cases and 155.000 deaths worldwide each year (Verissimo et al., 2018). Generally, *S.* Enteritidis, *S.* Infantis, *S.* Typhimurium, *S.* Heidelberg, and *S.* Corvallis are commonly linked to salmonellosis outbreaks notified in Europe, United States and Brazil (European Center for Disease Prevention and Control, 2017; Brazilian National Health Surveillance Agency, 2016; Center for Disease and Prevention, 2018).

Data from outbreaks investigation and surveys of industry premises indicate that food processing devices and environments might be the primary source of *S. enterica* (Podolak et al., 2010; Carrasco et al., 2012; Møller et al., 2016). The occurrence and persistence of *S. enterica* in food processing premises may be partially explained by its ability to attach and form biofilms in a variety of materials (O'Leary et al., 2015; Dhowlaghar et al., 2018; Iliadis et al., 2018). In the earlier stage of the biofilm formation, defined as adhesion, planktonic cells interact with the contact surface through weak chemical bonds. As such, the attached bacterial community can be removed by applying minimal forces (Bridier et al., 2015; Li et al., 2017). Nonetheless, the biofilm formation takes place when adhered cells become connected to the surface by hydrophobic interactions, covalent and ionic bonds and due to the production of exopolysaccharides (EPS) (Nguyen et al., 2014). After that, there is an increase in the population density and a pronounced production and deposition of EPS, increasing the attachment forces to the surface (Merino et al., 2017; Lamas et al., 2018). At this stage, the

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https://doi.org/10.1016/j.fm.2019.04.012
Received 10 February 2019; Received in revised form 31 March 2019; Accepted 25 April 2019
Available online 04 May 2019
0740-0020/

biofilm is mature, and the penetration of antimicrobial substances such as, chlorine-based disinfectants or quaternary ammonium, which are the most widely used agents for disinfection of food-contact surfaces is reduced and they are ineffective to cause the detachment of cells from the deeper biofilm layers (Hori and Matsumoto, 2010; Nguyen et al., 2014; Yang et al., 2016; Iñiguez-Moreno et al., 2018). As a result, cell will survive to cleaning approaches and will be further released from the mature biofilms leading to food recontamination, also known as cross-contamination (Ribaudo et al., 2017).

Stainless steel comprises the most common material used in industrial and domestic food-contact surfaces (Moraes et al., 2018) and the probability of adhesion and biofilm formation by *S. enterica* in this surface has been recently characterized (Moraes et al., 2018). However, various studies have reported that the irregularities of stainless-steel surface, as well as its corrosion by chemical disinfection procedures, may favor the accumulation of cells and increase the risks of adhesion and biofilm formation by *S. enterica* (Awad et al., 2012; Merino et al., 2017). Consequently, stainless steel has been replaced, for specific applications, by alternative materials, primarily polypropylene and glass (Carrasco et al., 2012; Srey et al., 2013).

Polypropylene and glass are used in domestic or industrial food-processing utensils, such as cutting boards, jars and tubs (Fink et al., 2017). Particularly, glass has been largely used to replace wooden cutting boards because wood is known to be a porous material, which is difficult to disinfect (Aviat et al., 2016; Fink et al., 2017). Glass is characterized as a hydrophilic material, thus presenting physical-chemical properties that may hinder cell attachment to surface (Donlan and Costerton, 2002). On the other hand, polypropylene is a low-cost light material with high hydrophobicity, a characteristic that facilitates bacterial adhesion (Abdallah et al., 2014; De Oliveira et al., 2014; Vidács et al., 2018). Because the increasing use of polypropylene and glass in food processing and preparation, studies have assessed the adhesion or biofilm formation by *S. enterica* on glass under different temperatures (De Oliveira et al., 2014) or growth media (Li et al., 2017). Besides, works have also evaluated the ability of *S. enterica* to attach or form biofilms under different growth conditions on polypropylene surfaces (Abdallah et al., 2014; Lianou and Koutsoumanis, 2012; Díez-García et al., 2012).

The adhesion and biofilm formation by *S. enterica* can be influenced by several variables related to the texture or roughness, hydrophobicity and chemical composition of the surfaces (Nguyen et al., 2014). However, biofilm-forming ability of this pathogen is mainly related to cell surface characteristics which are correlated with adhesion and surface colonization. Thus, the properties of the fluid around the surface (e.g.: water activity, pH, temperature, osmolarity, nutrients availability), which impact on the properties of bacterial cell surface, are also related to biofilm stages (Abdallah et al., 2014; Nguyen et al., 2014; Wakai and Harayama, 2015; Dhowlaghar et al., 2018).

Nevertheless, the impact of pH, temperature and NaCl concentration on the biofilm formation stages of epidemic *S. enterica* serovars on glass and polypropylene surfaces found during food processing have not been reported yet.

Given the above, understanding of the impact of environmental factors on the stages of biofilm formation is critical and quantitative approaches should be employed which will further allow the development of effective controlling strategies. Predictive models have been used as suitable tools to quantify bacterial behavior in foods (Dimakopoulou-Papazoglou et al., 2016). Predictive approaches have been employed to predict the boundaries of adhesion and biofilm formation by *S. enterica* on stainless steel through binomial logistic regression (Moraes et al., 2018). Other works also studied adhesion and biofilm formation (Møller et al., 2011; Pin et al., 2011), but the stages of biofilm formation were not assessed in the same model, despite their causal relationship. In this study, models to predict the biofilm formation stages on polypropylene and glass surfaces by *S. enterica* strains belonging to five prevalent serovars involved in food outbreaks were developed, for the first time, using ordinal logistic regression. The ordinal logistic regression has been primarily applied on population studies concerning to incidence and prevalence of comorbidities and their relationship with lifestyle, diet, socioeconomic aspects and risk factors (Das and Rahman, 2011; Södergren et al., 2012; Skropanic et al., 2016; Hageman et al., 2018). The analysis of ordinal regression allows to discriminate the answers in ordered categories or stages, which present a dependence or causal relationship between one or more independent variables as it occurs in biofilm formation (Abreu et al., 2009; Das and Rahman, 2011).

2. Materials and methods

2.1. S. enterica strains

Five *S. enterica* strains (*S.* Enteritidis 132, *S.* Infantis 176, *S.* Typhimurium 177, *S.* Heidelberg 281 and *S.* Corvallis 297) isolated from foods involved in salmonellosis outbreaks (Reference Collections of epidemic *Salmonella* strains of Central Laboratory of the Paraná State, LACEN-Paraná, Brazil) previously characterized as strong biofilm-producers by Moraes et al. (2018) were included in the study.

Stock cultures of the *S. enterica* strains were maintained in cryovials at −80 °C. The inoculum of each strain was obtained from cultures grown overnight in Tryptic Soy Broth (TSB; HiMedia, Mumbai, India) at 37 °C according to previously described (Melo et al., 2017).

2.2. Test surfaces

The polypropylene coupons of neutral color and low density (DWGA; Industrial Plastics, São Paulo, Brazil) and tempered glass coupons (VISA, Glass Security, Santa Catarina, Brazil), were used as experimental surfaces. Before the assays, the coupons (2 × 2 × 0.2 cm) were individually cleaned by immersion in alkaline detergent at 40 ± 2 C° (30 mL/1 L in distilled water; AUDAX, São Paulo, Brazil). Then, they were sanitized with 70% alcohol (CicloFarma, São Paulo, Brazil) and sterilized by autoclaving (121 °C for 15 min) using a described procedure by Rassoni and Gaylarde (2000).

2.3. Development of models for prediction of biofilm formation by S. enterica on surfaces

2.3.1. Experimental conditions

A total of 960 different (480 for each surface) combinations of pH values (4, 5, 6 and 7), NaCl concentration (0%, 2%, 4%, 6%, 8%, 10% w/v) and temperature (8 °C, 12 °C, 20 °C and 35 °C) were evaluated (Supplementary Table 1). The NaCl concentrations assayed corresponded to water activity (a_w) as follow: 0% NaCl (0.997), 2% NaCl (0.988), 4% (0.978), 6% NaCl (0.966), 8% NaCl (0.955) and 10% NaCl (0.940). Both pH and a_w values were selected considering the range of meat, poultry, fish and dairy products. The range of temperatures tested varied from the cold temperature usually applied during processing meat and dairy products in cold chain (Mercier et al., 2017) to a condition of high probability of biofilm formation because this comprises growth-favoring condition (International Commission on Microbiological Specifications for Foods - ICMSF, 1996; De Oliveira et al., 2014). The NaCl concentrations and pH values were attained in the medium following previously described procedures (Dimakopoulou-Papazoglou et al., 2016).

2.3.2. Evaluation of biofilm formation on polypropylene and glass surfaces by S. enterica

The lack of adhesion, adhesion and biofilm formation on polypropylene (PP) and glass (G) surfaces by each *S. enterica* strain was assessed by enumerating viable sessile cells. Two coupons of PP or G were immersed in sterile Petri dishes (60 × 15 mm) containing nine mL of TSB (with pH, NaCl, and temperature as indicated in Supplementary

Table 1) and one mL of the bacterial suspension (approximately 6 log CFU/mL). The surfaces were incubated for 72 h to allow the formation of mature biofilm (Yang et al., 2016). Afterward, the coupons were removed from the culture medium, washed with a sterile saline solution to remove planktonic cells and submitted to ultrasound (40 ± 2 kHz, 5 min) (Moraes et al., 2018). The obtained suspension was vortexed for 1 min, serially diluted in the same diluent and 20 µL aliquots of each dilution were spread-plated onto TSA using the micro drop inoculation technique (Herigstad et al., 2001). The plates were incubated at 35 °C for 24 h, and the results were expressed as log CFU/cm^2. The detection limit of the test was 1 log CFU/cm^2.

Biofilm stages were classified as follow: counts < 3 log CFU/cm^2 indicated lack of adhesion and were assigned to "category 1", counts ≥3 and < 5 log CFU/cm^2 corresponded to adhesion and were assigned to the "category 2". Finally, the counts ≥5 log CFU/cm^2 corresponded to biofilm formation and were assigned to "category 3" (Corcoran et al., 2014).

2.4. Development of ordinal logistic regression for biofilm formation by S. enterica

After the classification of results in categories based on enumeration of viable sessile *S. enterica* cells (i.e., category 1, 2 or 3), univariate analysis were performed by crossing each predictor (pH, NaCl concentration and temperature) with dependent variables on PP- and G-surfaces, using the Wald chi-square at significance level of P ≤ 0.05. Multiple regressions were performed to control possible confounding factors on the probability responses, and ordinal regression was used to assess the effects of predictor variables and their interactions (pH, NaCl concentration, temperature, surface material) on biofilm stages by each *S. enterica* strain.

Three ordinal responses were considered in the ordinal logistic regression the possibility as follows lack of adhesion ($Y = 1$), adhesion ($Y = 2$) and biofilm formation ($Y = 3$). Consequently, the categories were defined as $\alpha_1 < \alpha_2 < \alpha_3$, where the value of Y corresponds to the categories. Thus, the following responses are observed for Y:

$$Y = \begin{Bmatrix} 1 \text{ if } Y \le \alpha_1 \\ 2 \text{ if } \alpha_1 \le Y \le \alpha_2 \\ 3 \text{ if } \alpha_3 \le Y \le \infty \end{Bmatrix}$$

Based on that, the probability for each biofilm stage was calculated:

$$\pi_1 = P(Y = 1) = P(Y\alpha_1 \le 1) = P(x\beta + \varepsilon \le \alpha_1)$$

$$\pi_2 = P(Y = 2) = P(\alpha_1 \le y \le \alpha_2) = F(\alpha_2 - x\beta) - F(\alpha_1 - x\beta)$$

$$\pi_3 = P(Y = 3) = P(\alpha_2 \le y \le \alpha_{23}) = F(\alpha_3 - x\beta) - F(\alpha_2 - x\beta)$$

where Y is the variable response and π the biofilm stages (1: lack of adhesion; 2: adhesion; and 3: biofilm formation) and the predictors defined by pH, NaCl concentration, temperature and material of surface. The biofilm stages (Y) considered the independent variable with probabilities p1, p2 and p3 or $p_j = P(Y = j)$, for j = 1, 2, 3.

As values of the dependent variable denote an order of biofilm formation, then the ordinal logistic regression model with multiple (pH,NaCl, T, G, PP, pH × NaCl, pH × T, pH × G, pH × PP, NaCl × T, NaCl × G, NaCl × PP, T× G, T× PP, pH × NaCl × T, pH × NaCl × G, pH × NaCl × PP, pH × T× G, pH × TxPP, NaCl × T× G, NaCl × T× PP predictors can be written as:

$$\begin{aligned} \text{logit}[P(Y= j)] = {} & \alpha j - \beta 1\, pH + \beta 2 \times NaCl + \beta 3 \times T + \beta 4 \times G + \beta 5 \times PP \\ & + \beta 6 \times pH \times NaCl + \beta 7 \times pH \times T + \beta 8 \times pH \times G \\ & + \beta 9 \times pH \times PP + \beta 10 \times NaCl \end{aligned} \quad (1)$$

where Yj = prob(score ≤ j)/prob(score > j), αj (1, 2 or 3) is the intercept for the logit j (1, 2 or 3), and βn is the regression coefficient for the independent variable n = 21. The Wald's statistic, obtained as the square of the ratio of the coefficient to its standard error, was used to check whether the βn coefficients (P < 0.05) differ from zero.

All experiments were performed in triplicate, and the data were analyzed with the Statistical Software XLSTAT 2010 (software for Microsoft© Windows™ OS) by selection maximization of the likelihood function using the Newton-Raphson algorithm. The performance of each obtained model was evaluated with the McFadden and Nagelkerke R^2 statistic. The predictive power of the model was evaluated with the receiver Akaike's Information Criterion (AIC) and likelihood (−2 Log) of correctly described outcomes (classification of the predicted and observed biofilm stages).

3. Results

3.1. Biofilm formation on polypropylene and glass surfaces by S. enterica

Considering all tested combinations, 49.0% (647/960) of the outcomes corresponded to lack of adhesion (category 1). From the remained combinations, 15.0% (147/960) resulted in adhesion (category 2) and 14.0% (137/960) in biofilm formation (category 3) (Table 1). The evaluation of the outcomes considering the surface material showed that on PP-surface 73.0% (350/480) corresponded to lack of adhesion (category 1), while 13.0% (63/480) and 14.0% (69/480) of the combinations outcomes resulted in adhesion (category 2) and biofilm formation (category 3), respectively (Table 1). On the other hand, 67.0% (324/480) of the outcomes on G-surface corresponded to lack of adhesion (category 1), 17.7% (85/480) to adhesion (category 2) and 14.8% (71/480) to biofilm formation (category 3) (Table 1).

The ability to adhere or to form a biofilm on PP- or G-surface under the same environmental conditions varied among the *S. enterica* strains tested (Table 1). Under the food processing conditions tested, the highest number of outcomes for the lack of adhesion was observed for *S.* Heidelberg 281, while *S.* Typhimurium 177 showed the highest ability to adhere and *S.* Infantis 176 showed the highest ability to form a biofilm on both PP- and G-surface (Table 1).

3.2. Development of ordinal logistic regression for biofilm formation on polypropylene and glass surfaces by S. enterica

The ordinal regression models generated based on experimental data considered only the variables and interactions that presented significant effects (P < 0.05), however, a non-significant individual term was still kept in the model if it was involved in a significant interaction (Bursac et al., 2008). Notably, the predictor variable G-surface was excluded from the models because when the surface material was considered as a predictor, only PP-surface showed coefficient values different from zero (P < 0.05).

The goodness-of-fit statistics and the predictive power of the generated models using ordinal logistic regression obtained for each *S. enterica* strain are presented in Table 2. The Nagelkerke R^2 values of the models were ≥0.70, except for the model for *S.* Heidelberg 281. The values of Akaike's Information Criterion (AIC) statistic varied from 170.11 to 197.77 and the −2 Log (Likelihood) varied from 138.11 to 165.77 (Table 2). Overall, the percentage of biofilm stages correctly predicted by models was close to 70.0% for *S.* Typhimurium 177 and *S.* Corvalis 297 and varied from ~53 to 63% for the other strains tested (Table 2).

The estimated parameters selected from the ordinal logistic regression analysis are shown in Table 3. The strength of the effects caused by each parameter (pH, NaCl concentration, temperature, and surface) and their interactions on biofilm stages, based on Wald test varied among the five *S. enterica* strains tested (P < 0.05). For *S.* Enteritidis 132, *S.* Heidelberg 281 and *S.* Corvallis 297, the NaCl concentration caused more significant effects than temperature or pH on dependent variables (biofilm stages). On the other hand, for *S.* Infantis 176 and *S.* Typhimurium 177 temperature caused more significant

Table 1
Description of outcomes for the ordination of biofilm stages by five *S. enterica* strains belonging to different serovars on polypropylene and glass surfaces.

Categories Biofilm stages[a]	*S.* Enteritidis 132			*S.* Infantis 176			*S.* Typhimurium 177			*S.* Heidelberg 281			*S.* Corvallis 297		
	Overall	PP	G	Overall	PP	G	Overall	PP	G	Overall	PP	G	Overall	PP	G
1	141(73.4)	73(76.0)	68(70.8)	129(67.2)	66(68.7)	63(65.6)	127(66.5)	67(69.8)	60(62.5)	149 (77.6)	77(80.2)	72(75.0)	128(66.7)	67(69.8)	61(63.5)
2	31 (16.1)	14(14.6)	17(17.7)	25(13.0)	9(9.4)	16(16.7)	41 (21.5)	18(18.7)	23(23.9)	20(10.4)	10(10.4)	10(10.4)	31 (16.1)	12(12.5)	19(19.8)
3	20 (10.4)	9 (9.4)	11(11.5)	38 (19.8)	21(21.9)	17(17.7)	23 (12.0)	10(10.4)	13(13.5)	23 (11.9)	9 (9.4)	14(14.6)	33(17.2)	17(17.7)	16(16.7)

[a] Results presented as number of experiments and corresponding percentage [n (%)]; Overall: total number of outcomes observed on PP-surface and G-surface; PP: total number of outcomes observed on polypropylene surface; G: total number of outcomes observed on glass surface.

effects than pH or NaCl concentration (Table 3). Despite the lack of effects of the G-surface ($P \geq 0.05$), the PP-surface showed interaction with pH, NaCl concentration and temperature ($P < 0.05$) for all *S. enterica* strains tested (Table 3).

Considering the strength of the effects caused by the interaction of the predictor variables studied on biofilm stages, NaCl concentration × temperature × PP-surface caused the most potent effects for *S.* Enteritidis 132 and *S.* Corvallis 297. On the other hand, pH × temperature × PP-surface showed the most substantial effects for *S.* Infantis 176 and pH × temperature caused the most potent effects for *S.* Typhimurium 177. Finally, NaCl concentration × PP-surface showed the most substantial effects for *S.* Heidelberg 281 (Table 3).

Based on the generated model for each *S. enterica* strain the probability (Pr) of lack of adhesion or biofilm stages (adhesion or biofilm formation) was predicted as a function of the pH, NaCl concentration and temperature for PP-surface (Supplementary Tables 2–6) and G-surface (Supplementary Tables 7–11).

3.2.1. Probability of biofilm formation on PP-surfaces by ordinal logistic regression models

According to the generated models, the probability of adhesion and biofilm formation on PP-surface by the five *S. enterica* strains decreased in NaCl concentrations > 4% and temperature < 20 °C, at pH 4 or 5 (Supplementary Tables 2–6). However, at pH 5, in the absence or 2% NaCl and temperatures < 20 °C, *S.* Typhimurium 177 showed ~50% of adhesion (Supplementary Table 4).

At pH 4, in the absence of NaCl at 35 °C, *S.* Enteritidis 132 showed a probability of biofilm formation of 80% (Supplementary Table 2). Under these same conditions, *S.* Infantis 176 showed a probability of biofilm formation of 42.9%, while the probability observed for *S.* Heidelberg 281 and *S.* Corvallis 297 was 52.7 and 54.5%, respectively (Supplementary Tables 3, 5 and 6). At the same pH, in concentrations of NaCl > 4%, regardless of the temperature, the five strains showed a probability of ≥90% for lack of adhesion (Supplementary Tables 2–6). Interestingly, at pH 4, in the absence of NaCl and temperatures of ≥20 °C *S.* Typhimurium 177 showed a probability of adhesion around 60%, while the probability of biofilm formation was < 9.2% (Supplementary Table 4). Under the same conditions of pH in NaCl concentrations > 2% and at 35 °C, the probability of biofilm formation was reduced to 0% for *S.* Enteritidis 132, *S.* Infantis 176, *S.* Typhimurium 177 and *S.* Corvallis 297 (Supplementary Tables 2, 3, 4 and 6). Otherwise, *S.* Heidelberg 281, under these same conditions, showed 3.4% of the probability of biofilm formation (Supplementary Table 5). *S.* Enteritidis 132 and *S.* Typhimurium 177 showed a probability of adhesion > 50% at pH 4, in absence NaCl and at 35 °C (Supplementary Tables 2 and 4).

At pH 5, in absence NaCl and at 35 °C, *S.* Enteritidis 132 and *S.* Heidelberg showed a probability of biofilm formation of > 65%, while for *S.* Typhimurium 177 the probability of biofilm formation was 1.7% (Supplementary Tables 3, 4 and 5). Overall for *S.* Infantis 176 and *S.* Corvallis 297, under these same conditions the probability of biofilm formation was 100% (Supplementary Tables 3 and 6). At the same pH, in the absence of NaCl concentration, but at 20 °C, *S.* Typhimurium 177 showed 72.2% of the probability of biofilm formation. For this strain, at pH 5, in the absence of NaCl, the probability of adhesion remained > 50% even at 8 °C (Supplementary Table 4).

S. Infantis 176 and *S.* Corvallis 297 showed probability of biofilm formation > 90% at pH 5 in 2% NaCl and 35 °C (Supplementary Tables 3 and 6), while at the same pH and temperature, but in 4% NaCl these strains and *S.* Enteritidis 132 presented probability of adhesion around 40–50% (Supplementary Tables 2, 3 and 6). *S.* Infantis 176 and *S.* Corvallis 297 at pH 5, in 4% NaCl and at 35 °C showed a probability of biofilm formation around ~45% (Supplementary Tables 3 and 6). However, at pH 5 in NaCl concentrations > 4% and temperatures < 20 °C, all strains evaluated showed a probability of ≥90% for lack of adhesion (Supplementary Tables 2–6).

Table 2
Goodness-of-fit statistics and predictive power for the ordinal regression models to predict biofilm stages by *Salmonella enterica* strains belonging to different serovars.

Parameter	*S.* Enteritidis 132	*S.* Infantis 176	*S.* Typhimurium 177	*S.* Heidelberg 281	*S.* Corvallis 297
R^2 (Nagelkerke)	0.70	0.76	0.70	0.56	0.73
−2 Log (Log-likelihood)	138.11	146.72	165.77	158.73	158.09
AIC[a]	170.11	180.75	197.77	190.73	190.09
% predicted correctly	***S.* Enteritidis 132**	***S.* Infantis 176**	***S.* Typhimurium 177**	***S.* Heidelberg 281**	***S.* Corvallis 297**
Lack of adhesion	96.45	96.09	92.91	100.0	94.53
Adhesion	41.94	36.00	51.22	10.0	41.94
Biofilm	60.00	81.58	64.22	47.83	69.70
Total	63.13	53.42	69.78	52.61	68.72

[a] Akaike's Information Criterion (AIC).

When the conditions tested were pH 6 in the absence of NaCl and 35 °C, *S.* Heidelberg 281 showed a probability of biofilm formation of 75.8% (Supplementary Table 5). Under the same conditions, *S.* Enteritidis 132, *S.* Infantis 176 and *S.* Corvallis 297 showed probability ~100% of biofilm formation (Supplementary Tables 2, 3 and 6) and *S.* Typhimurium 177 showed 80.6% of probability of lack of adhesion (Supplementary Table 4). At pH 6, in 2% NaCl and at 20 °C, *S.* Corvallis 297 showed probability of biofilm formation around 60% (Supplementary Table 6), while *S.* Infantis 176 and *S.* Typhimurium 177, showed 92.9% and 85.6% of the probability of biofilm formation, respectively (Supplementary Tables 3 and 4). On the other hand, under these same conditions, *S.* Enteritidis 132 showed a probability of adhesion around 50% (Supplementary Table 2). Interestingly, at pH 6 and 35 °C, even with an increase of NaCl to 6%, *S.* Typhimurium 177 showed 72% of the probability of biofilm formation (Supplementary Table 4).

S. Enteritidis 132 showed probability of biofilm formation ≥98.1% at pH 7, in absence or 2% NaCl at 35 °C, while *S.* Infantis 176 and *S.* Corvallis 297 showed probability of biofilm formation of 100% at the same pH, in NaCl concentrations up to 4% even at 20 °C (Supplementary Tables 2, 3 and 6). *S.* Heidelberg 281 showed 84% of probability of biofilm formation at pH 7, in the absence of NaCl and 20 °C (Supplementary Table 5), while under the same conditions of pH and temperature, *S.* Enteritidis 132 showed a probability of adhesion around 50% even in NaCl concentration up to 4%. This strain showed the probability of adhesion of 55.7% at pH 7 and temperature 35 °C, even at 6% NaCl (Supplementary Table 2). Mainly, *S.* Typhimurium 177 showed a probability of adhesion around 50% at pH 7, in the absence of NaCl at temperatures < 12 °C (Supplementary Table 4).

3.2.2. Probability of biofilm formation on G-surfaces by ordinal logistic regression models

On G-surface, pH values ≥ 6, the absence of NaCl and temperatures < 20 °C increased the probability of adhesion by the *S. enterica* strains tested, except for *S.* Heidelberg 281. However, the probability of biofilm formation increased at pH 6 and 7, in NaCl concentrations up to 4% and temperatures 35 °C.

At pH 4, in the absence of NaCl and at 35 °C, *S.* Enteritidis 132 showed 74.8% of probability of biofilm formation, while *S.* Infantis 176 showed a probability of biofilm formation > 90% (Supplementary Tables 7 and 8). Under these same conditions, *S.* Typhimurium 177, *S.* Heidelberg 281 and *S.* Corvallis 297 showed a probability of biofilm formation of, 33.5%, 11.4%, and 55.4%, respectively (Supplementary Tables 9, 10 and 11). Otherwise, at pH 4 and 35 °C, but in 2% NaCl, *S.* Enteritidis 132, *S.* Infantis 176, *S.* Typhimurium 177 and *S.* Corvallis 297 showed a probability of adhesion was > 50%, while *S.* Heidelberg 281 of 17.5% (Supplementary Tables 7–11). Overall, at pH 4, in concentrations of NaCl > 4% and temperatures ≤20 °C, all *S. enterica* strains tested showed ≥90.0% of probability for lack of adhesion. When the conditions tested were pH 5 in the absence of NaCl, and at 35 °C, the probability of biofilm formation by *S.* Enteritidis 132, *S.* Infantis 176, *S.* Typhimurium 177 and *S.* Corvallis 297 was > 80% (Supplementary Tables 7, 8, 9, and 11). Under the same conditions, *S.* Heidelberg 281 showed 69.4% of the probability of biofilm formation (Supplementary Table 10). At pH 5, in absence of NaCl and at temperatures ≤20 °C, *S.* Typhimurium 177 and *S.* Corvallis 297 showed probability of adhesion ≥50%, while *S.* Infantis 176 and *S.* Enteritidis 132, showed probability of adhesion up to 50% in the same pH and NaCl conditions, but only in temperatures ≤12 °C (Supplementary Tables 7 and 8). On the other hand, under the same pH in the absence of NaCl, *S.* Heidelberg 281 showed a probability of adhesion < 20%, regardless of the temperature evaluated (Supplementary Table 10). Furthermore, at pH 5 in 6% NaCl and temperatures > 20 °C, *S.* Enteritidis 132, *S.* Infantis 176 and *S.* Typhimurium 177 showed a probability of adhesion around 50% (Supplementary Tables 7, 8 and 9). Otherwise, under the same conditions of pH, in NaCl concentration > 4% and temperatures < 20 °C, the five *S. enterica* strains tested showed probability > 95% for lack of adhesion (Supplementary Tables 7–11).

At pH 6 in the absence of NaCl and at a temperature < 20 °C, all strains tested, except for *S.* Heidelberg 281, showed a probability of adhesion around 50%, (Supplementary Table 10). Under the same pH, in the absence or 2% NaCl, but at a temperature > 20 °C, all strains, except for *S.* Infantis 176, presented probability of biofilm formation > 80.0% (Supplementary Tables 7–11). Otherwise, at pH 6 in 4% NaCl and 35 °C, *S.* Enteritidis 132 showed 60% of probability of adhesion, while the same probability of adhesion was observed for *S.* Typhimurium 177 under the same pH value and NaCl concentration, but at 20 °C (Supplementary Tables 7 and 9). Overall, at pH 6 in 4% NaCl, *S.* Infantis 176, *S.* Heidelberg 281 and *S.* Corvallis 297 presented probability of adhesion ~30% at temperatures ≥20 °C (Supplementary Table 8, 9 and 11). Notably, at pH 6 and at 35 °C, *S.* Corvallis 297 presented probability of adhesion around 50%, even at NaCl concentration of 8% (Supplementary Table 11), while *S.* Infantis 176 and *S.* Corvallis 297 showed 100% of probability for lack of adhesion at pH 6 in NaCl concentrations > 4% and temperatures < 20 °C (Supplementary Tables 8 and 11).

All strains showed probability > 90% of biofilm formation at pH 7, in 2% NaCl and at 35 °C (Supplementary Tables 7–11). At pH 7, in the absence of NaCl and temperatures < 20 °C, *S.* Enteritidis 132 and *S.* Typhimurium 177 showed a probability of adhesion around 60% (Supplementary Tables 7 and 9). Otherwise, under the same pH in NaCl concentrations, up to 4%, NaCl and temperatures > 20 °C, *S.* Enteritidis 132, *S.* Typhimurium 177 and *S.* Heidelberg 281 showed a probability of biofilm formation > 90% (Supplementary Tables 7, 9 and 10). *S.* Infantis 176 and *S.* Corvallis 297 showed a probability of adhesion around 50% at pH 7, in 4% NaCl and at 20 °C (Supplementary Tables 8 and 11). However, at pH 7, in NaCl concentrations > 4% and at temperatures < 20 °C, *S.* Enteritidis 132 and *S.* Typhimurium 177, showed a probability of adhesion and biofilm formation close to 0% (Supplementary Tables 7 and 9). Overall, at pH 7, NaCl concentration ≥6% and temperatures ≤20 °C, the five *S. enterica* strains showed around 90% of probability for lack of adhesion (Supplementary Tables 7–11).

Table 3
Estimates of parameters selected from the ordinal logistic regression analysis of predictors of biofilm stages by five *S. enterica* strains belonging to different serovars.

	Parameter value									
	S. Enteritidis 132		*S.* Infantis 176		*S.* Typhimurium 177		*S.* Heidelberg 281		*S.* Corvallis 297	
	Wald test	Value (CI)[a]	Wald test	Value (CI)	Wald test	Value (CI)	Wald test	Value (CI)	Wald test	Value (CI)
Constant 1	0.11	1.54 (−7.43, 10.5)	6.02	−12.43 (−11.36, −2.4.98)	0.00	0.17 (−8.16, 8.49)	2.66	12.43 (−2.49, 27.17)	0.78	1.20 (−7.17, 9.57)
Constant 2	0.94	4.48 (−4.57, 13.53)	0.13	1.57 (−6.96, 10.11)	0.06	3.32 (−5.03, 11.66)	3.32	13.81 (−1.05, 28.66)	0.70	3.59 (−4.82, 12.01)
Ph	0.00	−0.01 (−1.65, 1.64)	0.76	3.81 (−4.75, 12.37)	0.10	0.25 (−1.28, 1.78)	1.12	−1.33 (−3.68, 1.01)	0.06	−0.19 (−4.82, 12.01)
NaCl	2.43	3.24 (−0.84, 7.32)	0.00	0.01 (−1.53, 1.53)	0.98	1.32 (−1.28, 3.92)	3.85	−2.04 (−4.08, −0.002)	4.46	4.02 (0.29, 7.76)
T	0.00	0.01 (−0.43, 0.44)	6.88	−0.05 (−0.53, 043)	1.49	0.26 (−0.16, 0.67)	0.00	−0.01 (−0.56, 0.54)	0.04	0.04 (−0.34, 0.42)
Glass	–	–	–	–	–	–	–	–	–	–
PP	0.53	−4.27 (−15.78, 7.23)	0.88	−5.28(-16.30, 5.74)	2.03	−6.87(−16.32, 2.57)	3.96	−15.37 (−3.51, −0.24)	1.66	−7.53 (−18.98, 3.93)
pH × NaCl	0.98	−0.32 (−0.97, 0.317)	4.00	−0.37 (−0.73, −0.01)	0.26	−0.11 (−0.54, 0.32)	3.82	−0.35 (−0.001, 0.71)	2.77	−0.47 (−1.03, 0.08)
pH × T	0.93	−0.04 (−0.13, 0.04)	0.55	−0.03(0.12, 0.06)	4.45	−0.09 (−0.17, −0.01)	0.79	−0.05 (−0.14, 0.052)	0.77	−0.32 (−0.10, 0.04)
pH × Glass	–	–	–	–	–	–	–	–	–	–
pH × PP	1.22	1.18 (−0.91, 3.27)	2.90	1.75 (−0.26, 3.78)	2.43	1.35 (−0.35, 3.06)	3.22	2.22 (−0.20, 4.64)	4.92	2.49 (0.29, 4.69)
NaCl × T	2.60	−0.09 (−0.21, 0.02)	2.37	−0.07 (0.15, 0.02)	1.55	−0.56 (−0.14, 0.03)	1.04	0.04 (−0.04, 012)	3.07	−0.98 (−0.21, 0.01)
NaCl × Glass	–	–	–	–	–	–	–	–	–	–
NaCl × PP	2.19	−2.37 (−5.52, 0.77)	8.86	−3.61 (−5.98, −1.23)	0.44	−0.07 (−2.805, 1.38)	4.27	1.90 (0.09, 3.70)	7.27	−3.97 (−6.85, −1.08)
T × Glass	–	–	–	–	–	–	–	–	–	–
T × PP	2.25	0.42 (−0.13, 0.97)	10.01	1.03 (0.39, 1.67)	1.50	0.28 (−0.17, 0.72)	0.16	0.11 (−0.43, 0.65)	5.88	0.70 (0.13, −1.28)
pH × NaCl × T	2.44	0.02 (−0.004, 0.03)	2.77	0.01(-0.002–−0.03)	2.07	0.01 (−0.004, 0.03)	0.29	−0.004 (−0.02, 0.01)	2.47	0.01 (−0.003, 0.03)
pH × NaCl × Glass	–	–	–	–	–	–	–	–	–	–
pH × NaCl × PP	0.54	0.17(-0.28, 0.61)	6.03	0.44 (0.09, 0.79)	0.06	0.04 (−0.136, 0.04)	2.48	−0.26 (−0.59, 0.06)	3.93	0.39 (0.005, 0.79)
pH × T × Glass	–	–	–	–	–	–	–	–	–	–
pH × T × PP	2.37	−0.08 (−0.19, 0.02)	12.40	−0.25 (−0.38, −011)	1.14	−0.05 (−0.14, 0.04)	0.01	0.004 (−0.9, 0.09)	9.34	−0.19 (−0.32, −0.07)
NaCl × T × Glass	–	–	–	–	–	–	–	–	–	–
NaCl × T × PP	3.01	0.041 (−0.0, 0.09)	7.21	0.07 (0.02, 012)	1.01	0.02 (−0.02, 0.05)	0.22	−0.01 (−0.04, 0.026)	11.64	0.08 (0.035, 013)

[a] Confidence intervals - CI (95%); Glass: surface glass; PP: surface polypropylene; (−) the following variables do not bring significant information.

4. Discussion

The predictor factors selected for the evaluation of the biofilm stages in the present study mimic a broad range of environmental conditions applied as hurdles during food processing (Wang et al., 2013). The harsh environments resulting from food preservation strategies have been described as triggers for gene expression that enable planktonic cells to become sessile cells (Iliadis et al., 2018).

The ability to adhere and form biofilm under the environmental conditions assayed varied among the tested *Salmonella* strains. Previous studies assessing the biofilm formation ability of *S. enterica* strains isolated from pork processing plants (Castelijn et al., 2013) and chicken slaughter plant (Wang et al., 2013) reported that *S.* Typhimurium and *S.* Infantis showed a high capacity of biofilm formation. In the present study, *S.* Typhimurium 177 and *S.* Infantis 176 showed a greater ability of adhesion, and biofilm formation on PP-surface and G-surface compared to *S.* Enteritidis 132, *S.* Heidelberg 281 and *S.* Corvallis 297. Among the more than 2500 serotypes of *S.* Typhimurium and *S.* Infantis has been the prevalent serovars causing salmonellosis outbreaks worldwide (Alam et al., 2018; Arai et al., 2018).

Additionally, *S.* Infantis 176 and *S.* Typhimurium 177 showed greater ability to adhere and form a biofilm on acidic conditions (pH 4 and 5) and high NaCl concentration (up to 6%). These serovars have been described as high tolerant to thermal, acid and osmotic stress conditions used in traditional food preservation systems (Spector and Kenyon, 2012; Melo et al., 2017). This increased tolerance to stress conditions could be related to the great ability to form biofilms under the environmental conditions assayed in the present study. Studies have shown that when exposed to acid sanitizers, cold temperatures and osmotic pressure may increase the gene expression of extracellular components related to adhesion and biofilm formation (e.g. amyloid curli fimbriae and bacterially produced cellulose) in *S. enterica* serovars such as *S.* Enteritidis and *S.* Typhimurium (De Oliveira et al., 2014; Piras et al., 2015; Borges et al., 2018).

On G-surface, *S. enterica* strains tested showed a higher percentage of adhesion and biofilm formation than on PP-surface, except for biofilm formation by *S.* Infantis 176 (Table 1). These results are exciting because polypropylene has been cited as a surface that possesses hydrophobic nature, and thus may favor the bacterial adhesion (Donlan and Costerton, 2002; Abdallah et al., 2014; Vidács et al., 2018). However, the biofilm-forming abilities of foodborne pathogens are mainly related to cell surface characteristics which are correlated with adhesion and surface colonization, for instance, the adhesion ability is increased with increasing cell surface hydrophobicity (Xu et al., 2010; Wakai and Harayama, 2015; Iliadis et al., 2018). Since cell surface can be modulating by changes temperature, pH, NaCl, and other stress conditions it is possible that the exposure of *S. enterica* strains to the tested conditions increased cell surface hydrophobicity, thus the adhesion ability may be increased despite the hydrophobicity of G-surface.

Based on the extensive set of the experimental data, the ordinal regression models built were devoted to fit the categorical responses (lack adhesion, adhesion, and biofilm formation) by the five *S. enterica* strains predicted by pH, NaCl concentration and temperature. The Nagelkerke's-R^2 coefficient generally ≥ 70, showed the usefulness of the variables pH, NaCl concentration and temperature to predict the biofilm stages on PP- and G-surfaces (Table 2). This coefficient indicates how the model explains the effects of changes in independent variables, and values which explained the majority of results are regarded as positive (Veall and Zimmermann, 1992). The fact that the Log-likelihood statistic was in a range of 138.11–165.77 indicates their proper fitting for the five obtained models since low values indicate a bad fitting model (Rosseel, 2012). Otherwise, the lower AIC values obtained for models built for *S.* Enteritidis 132 and *S.* Infantis 176 indicate a better fit compared to models obtained for the other strains tested (Burnham and Anderson, 2002). Overall, the five ordinal models showed high ($> 90\%$) percentage of outcomes correctly predicted for lack of adhesion, showing their great predictive power for this category (Agresti and Hartzel, 2000). From the industrial point of view, the correct prediction of lack of adhesion is of great importance, considering the causal relationship of adhesion and biofilm formation. Therefore, if adhesion does not occur, biofilm formation will be prevented as well, and the models are useful tools to support the choice of preservation hurdles."

Furthermore, for ordinal regression models, few statistics are recognized to assess goodness of fit, and no general goodness-of-fit test is widely available in software packages (Fagerland and Hosmer, 2012). Overall, the deviance statistics is used for testing goodness of fit in ordinal models and show predicted correctly of dates and models (Pulkstenis and Robinson, 2004). Our results showed an adequate performance of the generated models to predict the lack of adhesion and biofilm formation categories but considering deviance statistics the prediction of the adhesion category did not show good fit. The space between adhesion and biofilm formation is almost imprecise since the formation of a microbial biofilm is influenced by many factors related to the environment and bacterial cell characteristics (Díez-García et al., 2012; Dimakopoulou-Papazoglou et al., 2016). Additionally, this may be related to the cell activation of distinct virulence factors to survive under stress conditions making hard a strict definition of these events as adhesion.

In the current study, the predictors pH, NaCl concentration and temperature, as well as their interactions affect the biofilm stages on PP- and G-surface similarly to observed by Moraes et al. (2018) in stainless steel surfaces. Among the predictor variables, the NaCl concentration caused more significant effects on biofilm formation stages of *S.* Enteritidis 132, *S.* Heidelberg 281 and *S.* Corvallis 297. However, the combination between the variables NaCl concentration, temperature and surface material showed the most potent effects on biofilm formation by *S.* Enteritidis 132 and *S.* Corvallis 297, while the interaction between pH and temperature caused the most potent effects on biofilm formation by *S.* Typhimurium 177 (Table 3).

Therefore, the increase or decrease of pH, NaCl concentrations and temperature affect the probability of lack of adhesion, adhesion and biofilm formation on PP- and G-surfaces, but the intensity of the effects varied among the *S. enterica* strains tested. The distinct strength of the effects caused by the predictors is probably related to intrinsic characteristics of the strains since distinct strains/serovars can show react in different ways to changes in the environmental conditions (Iliadis et al., 2018). Primarily, changes in environmental conditions can result in modification of the chemical composition of the bacterial cell surface and affect the presence and disposition of bacterial surface components such as flagella, fimbriae, and polysaccharide, which in turn are intrinsically related to adhesion processes and biofilm formation (Steenackers et al., 2012).

Only the reduction of pH and the absence of NaCl were not enough to reduce the probability of biofilm formation on G-surface at the ideal temperature of *S. enterica* growth (35 °C). Similar results were observed when these same *S. enterica* strains were tested for adhesion and biofilm formation on stainless steel surfaces as a function of pH, temperature, and NaCl concentration (Moraes et al., 2018). However, pH < 5, a temperature of 35 °C and in the absence of NaCl on PP-surface, *S.* Corvallis 297 showed a decrease of probability to $\sim 50\%$ of biofilm formation. These results reinforce the distinct ability of each strain tested to adhere or form biofilm because the reduction of pH affects bacterial cell through the mechanism of feedback. When a cell is exposed to a higher H^+ concentration in the environment, it directs the energy to eliminate H^+ cytoplasmic, which in turns reduce the capacity of adhesion and formation of biofilm (Dimakopoulou-Papazoglou et al., 2016).

Overall, under acid conditions pH (≤ 4), NaCl concentrations $> 2\%$ and at temperatures < 35 °C the probability of biofilm formation on PP- the surface was decreased to 0% for the five *S. enterica* strains

tested. These results highlight the interaction effect between pH, NaCl concentration and temperature. Additionally, at pH 5, higher NaCl concentration (> 4%) and temperatures < 20 °C there was a reduction of the probability of both adhesion and biofilm formation, except for *S.* Typhimurium 177 on PP-surface. The decrease of adhesion and biofilm could be explained because a decrease of pH promotes osmotic imbalance of the cell and elevation of osmotic pressure (Garrett et al., 2008) which was strengthened by the increase of NaCl concentration. Because *S. enterica* respond to osmolality changes by modulating the expression of different pools of genes, including those involved on adherence to surfaces (Tartera and Metcalf, 1993) probably the adhesion and consequently the biofilm formation was decreased. On the other hand, the ability of *S.* Typhimurium 177 to form a biofilm on PP-surface under acid and high osmolarity environment may be attributed to its ability to induce adaptive mechanisms upon exposures to these stresses (Gabriel et al., 2015).

Overall, a temperature of 20 °C decrease biofilm formation and increase adhesion in both PP-surface and G-surface. Various studies have investigated biofilm formation by *S. enterica* strains belonging to distinct serovars under different temperature conditions (Agarwal et al., 2011; Lianou and Koutsoumanis, 2012; Borges et al., 2018; Moraes et al., 2018). Generally, the results suggest that temperature near 20 °C prevent the formation of biofilm, but allows of adhesion, the stage that precedes the formation of biofilm. In general, it is considered that the decline of the temperature reduces or even pause bacterial enzymatic activity. Thus metabolism, multiplication, and adhesion to surfaces are minimized or prevented (De Oliveira et al., 2014; Giaouris et al., 2015; Melo et al., 2017). However, none studies have investigated the stages of biofilm formation in different combinations of environmental conditions and according earlier studies the exposure to temperatures < 30 °C may favor the adhesion and biofilm formation on plastic surfaces, because they promote the gene expression of extracellular components (Agarwal et al., 2011; Lianou and Koutsoumanis, 2012; Schonewille et al., 2012).

5. Conclusions

The ability to adhere or form a biofilm on PP- or G-surfaces under the environmental conditions assayed varied with the *S. enterica* strains tested. The ordinal regression models obtained for the five *S. enterica* strains showed an excellent performance to predict the adhesion and biofilm formation, described for the first time as ordered categories or stages. The models also highlight the variability among *S. enterica* strains belonging to the different serovars involved in outbreaks and previously characterized as stronger biofilm producers. The models built in this study may be useful tools for industrial application and on risk assessment studies because they describe the impact of pH, NaCl concentration and temperature applied during food processing on the biofilm formation stages by *S. enterica* strains. Finally, the generated models on simulated food processing conditions expand the understanding of the conditions that impact on biofilm formation steps in food-contact surfaces. This understanding is of paramount relevance because it may provide the basis for the implementation of more effective hygienic procedures aiming to safeguard public health.

Acknowledgments

M. Magnani thanks the financial support of *National Council for Scientific and Technological Development* (CNPq) (Grants #405644/2018-3 and #402472/2018-7, respectively). A. S. Sant'Ana acknowledges the support from National Council for Scientific and Technological Development (CNPq) for the financial support (Grants: #302763/2014-7 and #305804/2017-0). This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.04.012.

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