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Modelling biofilm formation of *Salmonella enterica* ser. Newport as a function of pH and water activity

Dafni Dimakopoulou-Papazoglou, Alexandra Lianou [1], Konstantinos P. Koutsoumanis [*]

Laboratory of Food Microbiology and Hygiene, Department of Food Science and Technology, School of Agriculture, Forestry and Natural Environment, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece

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ABSTRACT

The effect of pH and water activity (a_w) on the formation of biofilm by *Salmonella enterica* ser. Newport, previously identified as a strong biofilm producer, was assessed. Biofilm formation was evaluated in tryptone soy broth at 37 °C and at different combinations of pH (3.3–7.8) and a_w (0.894–0.997). In total, 540 biofilm formation tests in 108 pH and a_w combinations were carried out in polystyrene microtiter plates using crystal violet staining and optical density (OD; 580 nm) measurements. Since the individual effects of pH and a_w on biofilm formation had a similar pattern to that observed for microbial growth rate, cardinal parameter models (CPMs) were used to describe these effects. CPMs described successfully the effects of these two environmental parameters, with the estimated cardinal values of pH_{min}, pH_{opt}, pH_{max}, a_{wmin} and a_{wopt} being 3.58, 6.02, 9.71, 0.894 and 0.994, respectively. The CPMs assumption of the multiplicative inhibitory effect of environmental factors was validated in the case of biofilm formation using additional independent data (i.e. 430 OD data at 86 different combinations of pH and a_w). The validation results showed a good agreement ($r^2 = 0.938$) between observed and predicted OD with no systematic error. In the second part of this study, a probabilistic model predicting the pathogen's biofilm formation boundaries was developed, and the degree of agreement between predicted probabilities and observations was as high as 99.8%. Hence, the effect of environmental parameters on biofilm formation can be quantitatively expressed using mathematical models, with the latter models, in turn, providing useful information for biofilm control in food industry environments.

1. Introduction

In nature, the majority of bacteria are organized in surface-associated communities, known as biofilms. These communities are adhered onto surfaces, biotic or abiotic, and are enclosed within an extracellular polymeric matrix produced by the bacteria themselves (Branda et al., 2005; Steenackers et al., 2012; Wong and O'Toole, 2011). The main components of such a matrix are polysaccharides, proteins, nucleic acids and phospholipids (Sutherland, 2001; Wingender et al., 1999). Within biofilms, bacterial cells are in a different state compared to those growing planktonically, and are protected against various adverse environmental conditions such as UV light radiation, metal toxicity, pH and osmotic changes, dehydration, host immune responses, antimicrobial agents and disinfectants (Donlan and Costerton, 2002; Karunakaran et al., 2011; Nilsson et al., 2011; Smirnova et al., 2010). As such, bacterial biofilms constitute a significant concern, both for food safety authorities and the food industry where biofilms may be a major source of food contamination with spoilage and pathogenic microorganisms (Shi and Zhu, 2009; Stepanović et al., 2004).

Salmonella enterica, one of the most common causes of foodborne disease worldwide, is a bacterial species capable of adhering to and forming biofilms on both biotic and abiotic surfaces (Giaouris and Nychas, 2006; Joseph et al., 2001; Vestby et al., 2009). The adhesion and biofilm-forming ability of this pathogen depend on several factors including the growth medium, the growth phase of the cells, the type and properties of the inert material, the contact time, the presence of organic material, as well as environmental parameters such as temperature and pH (Speranza et al., 2011).

Due to its major public health significance, the complex and

* Corresponding author.
E-mail address: kkoutsou@agro.auth.gr (K.P. Koutsoumanis).
[1] Present address: Laboratory of Microbiology and Biotechnology of Foods, Department of Food Science and Human Nutrition, School of Food, Biotechnology and Development, Agricultural University of Athens, Athens 11855, Greece.

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multifactorial phenomenon of biofilm formation has been studied extensively the last decade. Indeed, the ability of pathogenic bacteria, including *S. enterica*, to form biofilms on various surfaces and under different environmental conditions has been investigated in several recent research studies (Bonsaglia et al., 2014; Díez-García et al., 2012; Lianou and Koutsoumanis, 2012; Nilsson et al., 2011). However, despite the widespread application of mathematical models for the prediction of microbial growth and survival, and the significant amount of scientific literature on modelling of biofilm processes (Beg and Chaudhry, 1999; Wanner et al., 2006), there has not been made any attempt until now to develop and implement mathematical models for the description of the effects of environmental factors on biofilm formation. Therefore, the objective of this study was the development and evaluation of predictive mathematical models for the description of the effects of pH and water activity on the biofilm-forming ability of *S. enterica*.

2. Materials and methods

2.1. Bacterial strain

The bacterial strain used in this study was *S. enterica* serotype Newport FSL R6-051 (bovine intestine isolate), kindly provided by Dr. Martin Wiedmann (Cornell University, Ithaca, NY, USA). Based on the findings of a previous study undertaken in our laboratory, this strain was characterized as a strong biofilm producer in a wide range of environmental conditions (Lianou and Koutsoumanis, 2012).

The stock culture of the strain was stored frozen (−70 °C) onto Microbank™ porous beads (Pro-Lab Diagnostics, Ontario, Canada), whereas the working culture was maintained at 5 °C on tryptone soy agar (TSA; Lab M Limited, Lancashire, UK) slant and was renewed bimonthly. The strain was activated by transferring a loopful from the TSA slant into 10 ml of tryptone soy broth (TSB; Lab M Limited) and incubating at 37 °C for 24 h.

2.2. Biofilm formation

2.2.1. Environmental conditions

The biofilm-forming ability of the tested *S.* Newport strain was evaluated in TSB at 37 °C, in high-precision (±0.2 °C) incubators (Sanyo MIR 153, Sanyo MIR 253, Sanyo Electic Co., Ora – Gun, Japan), and at different combinations of pH and water activity (a_w) values. More specifically, 11 pH values (3.3, 3.5, 3.8, 4.2, 4.6, 5.0, 5.4, 5.8, 6.5, 7.35 and 7.8) and 16 different NaCl concentrations (0.5%, 2.5%, 3.5%, 4.5%, 6.0%, 7.0%, 8.0%, 9.0%, 10.0%, 11.0%, 12.0%, 13.0%, 14.0%, 15.0%, 16.0% and 17.0% w/v) were evaluated. The pH of TSB was adjusted to the above values with HCl (min. 37%; Sigma–Aldrich, Seelze, Germany) using a digital pH meter with an epoxy refillable pH probe (Thermo Electron Corporation, Beverly, MA, USA). The abovementioned salt concentrations were achieved by adding to the medium (which has a NaCl concentration of 0.5% w/v) appropriate amounts of NaCl (Merck, Darmstadt, Germany). The a_w values of the substrates were measured with an Aqualab water activity meter (Model series 3; Decagon Devices, Inc., Pullman, WA, USA) and were the following: 0.997 (0.5% NaCl), 0.987 (2.5% NaCl), 0.980 (3.5% NaCl), 0.976 (4.5% NaCl), 0.966 (6.0% NaCl), 0.960 (7.0% NaCl), 0.955 (8.0% NaCl), 0.950 (9.0% NaCl), 0.940 (10.0% NaCl), 0.934 (11.0% NaCl), 0.928 (12.0% NaCl), 0.922 (13.0% NaCl), 0.916 (14.0% NaCl), 0.909 (15.0% NaCl), 0.903 (16.0% NaCl) and 0.894 (17.0% NaCl). The pH and a_w values of the substrates were also measured after sterilization in order to ensure that they were not considerably changed. Five replicates for each pH and a_w combination were tested resulting in a total of 540 tested samples from 108 pH and a_w combinations.

2.2.2. Biofilm formation assay

Biofilm formation was quantified using a colorimetric method which is based on the measurement of the optical density of biofilms developed in microtiter plate wells after crystal violet staining. Various versions of such method have been used for the *in vitro* evaluation of biofilm production (Agarwal et al., 2011; Díez-García et al., 2012; Naves et al., 2008; Stepanović et al., 2000, 2003, 2004), and the exact protocol implemented in this study for biofilm formation and quantification has been described in detail previously (Lianou and Koutsoumanis, 2012). Briefly, 20-μl aliquots of the 24-h *S. enterica* culture were added to 180 μl of TSB (with the characteristics described in 2.2.1) dispensed in 100-well polystyrene microtiter plates (Oy Growth Curves Ab Ltd., Raisio, Finland), while negative control wells (i.e. containing broth only) also were included. The microtiter plates were incubated statically at 37 °C for 48 h, and then the content of the plates' wells was discarded (removal of non-adherent or reversibly attached cells) and the wells were rinsed with sterile quarter strength Ringer's solution (Lab M Limited). The adherent bacterial cells were fixed with methanol (min. 99.8%; Scharlau Chemie S.A., Barcelona, Spain), and the plates' wells were then emptied, air-dried and stained with crystal violet (Gram's crystal violet solution; Merck). After rinsing off the excess stain with distilled water and air-drying of the microtiter plates, the crystal violet bound to the formed biofilms was solubilized in absolute ethanol (min. 99.8; Sigma–Aldrich), and the optical density of each well was measured at 580 nm using the turbidimetric system Bioscreen C (Oy Growth Curves Ab Ltd.). The quantification of biofilm formation was based on the difference between the optical density measurements of the test and negative control (uninoculated) samples (ΔOD) (Lianou and Koutsoumanis, 2012).

2.3. Model development

2.3.1. Modelling the extent of biofilm formation

In order to describe the individual effects of pH and a_w on the extent of biofilm formation, the collected ΔOD values at various pH conditions (from 3.8 to 7.8) at optimum a_w (0.997) and various a_w conditions (from 0.916 to 0.997) at optimum pH (6.5) were fitted to the cardinal parameter models of Rosso (Rosso et al., 1995) for pH and a_w, respectively:

$$\Delta OD = \Delta OD_{opt} \cdot \rho(pH)$$

$$\rho\,(\mathrm{pH}) = \begin{cases} 0, & \mathrm{pH} \le \mathrm{pH}_{min} \\ \dfrac{(\mathrm{pH}-\mathrm{pH}_{max})\cdot(\mathrm{pH}-\mathrm{pH}_{min})}{(\mathrm{pH}_{opt}-\mathrm{pH}_{min})\cdot(\mathrm{pH}-\mathrm{pH}_{opt}) - (\mathrm{pH}_{opt}-\mathrm{pH}_{max})\cdot(\mathrm{pH}_{min}-\mathrm{pH})}, & \mathrm{pH}_{min} < \mathrm{pH} < \mathrm{pH}_{max} \\ 0, & \mathrm{pH} \ge \mathrm{pH}_{max} \end{cases} \quad (1)$$

where pH_{min}, pH_{opt} and pH_{max} are the corresponding cardinal values, and ΔOD_{opt} is the ΔOD corresponding to pH = pH_{opt}.

$$\Delta OD = \Delta OD_{opt} \cdot \rho(a_w)$$

$$\rho(\alpha_w) = \frac{(a_w - 1)\cdot(a_w - 1)^2}{(a_{wopt} - a_{wmin})\cdot\left[(a_{wopt} - a_{wmin})\cdot(a_w - a_{wopt}) - (a_{wopt} - 1)\cdot(a_{wopt} + a_{wmin} - 2\cdot a_w)\right]} \quad (2)$$

where α_{wmin} and α_{wopt} are the corresponding cardinal values, and ΔOD_{opt} is the ΔOD corresponding to $a_w = a_{wopt}$.

For the purpose of model validation, the combined effect of pH and a_w on the ΔOD was predicted using the following equation:

$$\Delta OD = \Delta OD_{opt} \cdot \rho(pH) \cdot \rho(\alpha w) \quad (3)$$

For the ΔOD_{opt}, the average of the ΔOD_{opt} values estimated from equations (1) and (2) was used.

In the present study, the Relative Biofilm Formation Index (RBFI) was introduced as a more effective measurement for the quantification of the effect of the environment on the extent of biofilm formation. The RBFI is defined as the ratio of the ΔOD value derived from the crystal violet assay at a certain environmental condition and the maximum ΔOD value observed at the optimum condition for biofilm formation:

$$RBFI = \Delta OD / \Delta OD_{opt} \quad (4)$$

From equations (3) and (4), RBFI can be predicted by the CPM as follows:

$$RBFI = \rho(pH)\cdot\rho(\alpha w) \quad (5)$$

2.3.2. Modelling the boundaries of biofilm formation

The collected optical density data (ΔOD), corresponding to different environmental conditions, were converted into binary data (0: no biofilm formation, 1: biofilm formation), using a threshold RBFI value equal to 0.1 (10% ΔOD_{opt} observed at optimum conditions). Then, a logistic polynomial regression model was fitted to the binary data using Minitab ver. 13 (Minitab Inc., State College, PA, USA), based on the approach described by Ratkowsky and Ross (1995). The model's equation is shown below:

$$\text{Logit}(P) = \alpha_0 + \alpha_1\cdot pH + \alpha_2\cdot a_w + \alpha_3\cdot pH\cdot a_w + \alpha_4\cdot pH^2 + \alpha_5\cdot a_w{}^2 \quad (6)$$

where Logit (P) is an abbreviation of $\ln[P/(1-P)]$, P is the probability of biofilm formation (in the range of 0–1) and α_i are the coefficients to be estimated.

The automatic variable selection option with a stepwise selection method was used to choose the most significant effects ($P < 0.05$). The predicted biofilm formation/no formation interfaces for $P = 0.1$, 0.5 and 0.9 were calculated using Excel Solver (Microsoft Corp., Redmond, WA, USA).

3. Results and discussion

3.1. Modelling the extent of biofilm formation

In a previous study undertaken in our laboratory (Lianou and Koutsoumanis, 2012), the *S. enterica* strain used in the present study (*S.* Newport FSL R6-051) was found to be the strongest biofilm producer among 60 strains of the pathogen. In the context of the present study, the effects of pH and a_w on the biofilm formation ability of this strain were evaluated in TSB after 48 h of incubation in polystyrene microtiter plates. The extent of biofilm formation was quantified using crystal violet staining, and was based on the difference between the optical density measurements of the test and negative control (uninoculated) samples (ΔOD). The results showed that the individual effects of pH and a_w on biofilm formation, expressed in ΔOD values, had a similar pattern to that expected for microbial growth rate. For example, in the case of pH, an increase in the ΔOD values was observed as pH increased from 3.8 up to an optimum value followed by a decrease for pH values above this optimum (Fig. 1). Similarly, ΔOD values increased from $a_w = 0.916$ up to a maximum value of 0.997 (Fig. 2).

Based on the above observation, CPMs were used to describe the effects of pH (equation (1)) and a_w (equation (2)) on biofilm formation expressed in ΔOD. Since their initial introduction in predictive microbiology in 1993, CPMs have become an important group of empirical secondary models for growth rate and lag phase (Augustin and Carlier, 2000; Rosso and Robinson, 2001; Rosso et al., 1993, 1995). The main advantage of CPMs is that they are based on model parameters that have both biological and graphical interpretation. The results of the present study showed that CPMs are also appropriate for modelling the effect of pH and a_w on biofilm formation, with the latter being expressed in ΔOD values. As it is shown in Figs. 1 and 2, the applied secondary models described successfully the effects of these two environmental parameters on biofilm formation, with the estimated values of coefficient of

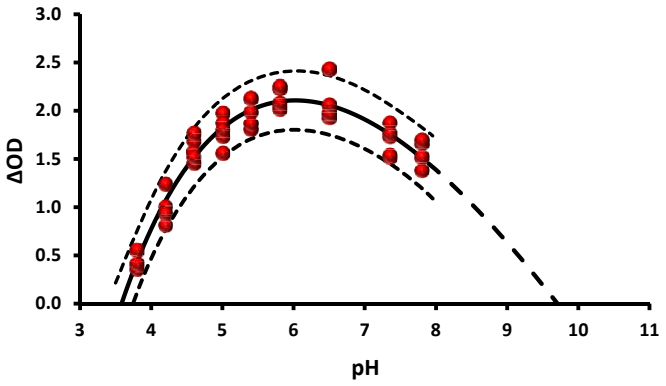

Fig. 1. Cardinal parameter model for the effect of pH (equation (1)) on biofilm formation, expressed in ΔOD values, of *Salmonella* enterica ser. Newport in tryptone soy broth with $a_w = 0.997$ at 37 °C. (Points: observed data; solid line: model fitting; dotted lines: 95% prediction intervals).

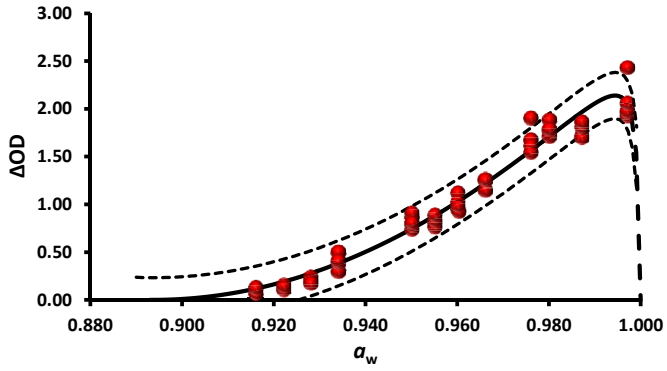

Fig. 2. Cardinal parameter model for the effect of a_w (equation (2)) on biofilm formation, expressed in ΔOD values, of *Salmonella enterica* ser. Newport in tryptone soy broth with pH = 6.5 at 37 °C. (Points: observed data; solid line: model fitting; dotted lines: 95% prediction intervals).

Table 1
Estimated values for the parameters and fitting statistics of the cardinal parameter model describing the effect of pH and a_w on ΔOD.

Parameter	Estimated value[a]	95% Confidence limits		r^{2b}	RMSE[c]
		Lower	Upper		
pH model					
pH_{min}	3.58 ± 0.05	3.48	3.69	0.931	0.139
pH_{max}	9.71 ± 0.36	8.98	10.44		
pH_{opt}	6.02 ± 0.07	5.88	6.17		
ΔOD_{opt}	2.107 ± 0.040	2.026	2.188		
a_w model					
a_{wmin}	0.894 ± 0.002	0.889	0.899	0.972	0.113
a_{wopt}	0.994 ± 0.001	0.993	0.995		
ΔOD_{opt}	2.139 ± 0.033	2.073	2.206		

[a] Values are means ± standard errors.
[b] r^2: Coefficient of determination.
[c] RMSE: Root Mean Square Error.

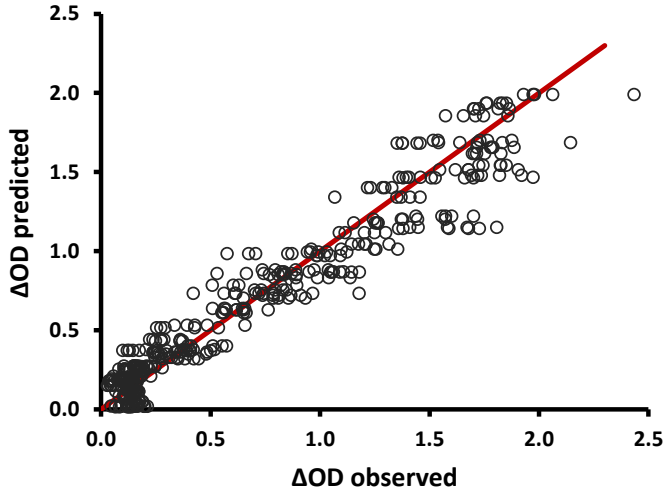

Fig. 3. Comparison between the predicted and observed ΔOD values for biofilm formation of *Salmonella enterica* ser. Newport at various combinations of pH and a_w derived from independent experiments.

determination (r^2) being 0.931 and 0.972 for pH and a_w, respectively, while the corresponding root mean square error (RMSE) values were 0.193 and 0.113 (Table 1). The estimated cardinal values of pH_{min}, pH_{opt}, pH_{max}, a_{wmin} and a_{wopt} were 3.58, 6.02, 9.71, 0.894 and 0.994, respectively, while the estimated optimum ΔOD values for pH and a_w were almost identical (Table 1).

General CPMs (equation (3)) rely on the assumption that the inhibitory effect of environmental factors is multiplicative, and thus, consist of a discrete term for each environmental factor. Each term ρ in equation (3) has a numerical value between 0 and 1 and at optimum conditions all terms have a value of 1. The successful use of many general CPMs models for microbial growth rate has shown this assumption to be reasonable for a wide range of environmental conditions. In this study, the validity of the abovementioned assumption in the case of biofilm formation (ΔOD data) was evaluated. For this, 430 ΔOD data on biofilm formation of *S.* Newport at 86 different combinations of pH and a_w ranging from 3.8 to 7.8 and from 0.894 to 0.987, respectively, were produced from independent experiments and were compared with the ΔOD predicted by the combined CPM model (equation (3)). The validation results are presented in Fig. 3. In general, a good agreement (r^2 = 0.938) between observed and predicted ΔOD was found, with no systematic error, supporting the validity of the assumption on multiplicative inhibitory effect of pH and a_w on biofilm formation. For low ΔOD values (<0.2) however, a high variability was observed among replicate samples at the same pH and a_w conditions. Such an observation could be attributed to the potential presence of low levels of excess dye even in samples with no biofilm formation, which may result in pigment and a slight increase in the optical density measurements.

The structural components of *S. enterica* biofilms include curli and other fimbriae, surface proteins, flagella, cellulose, colanic acid,

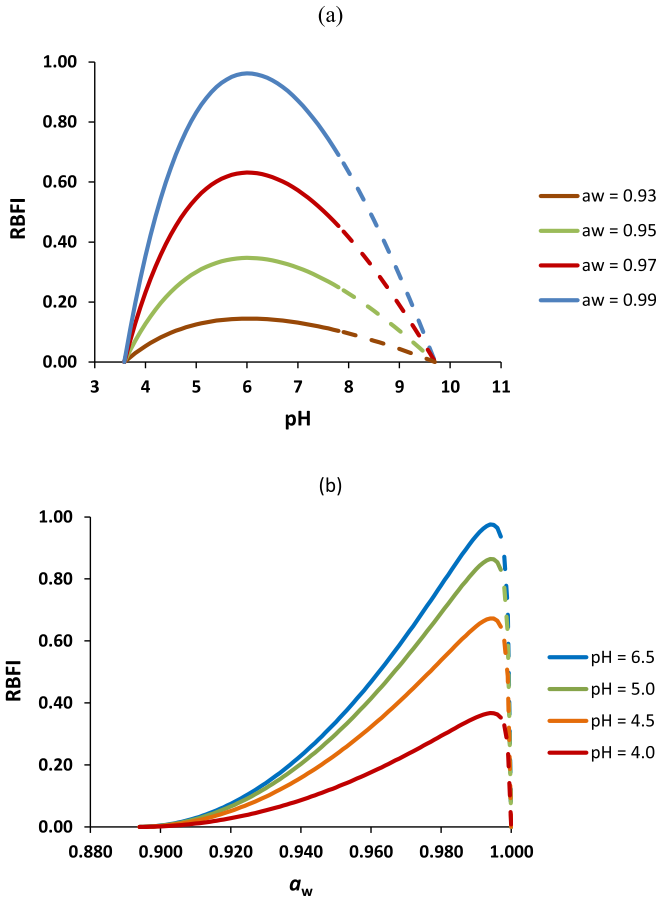

Fig. 4. Effect of pH (a) and a_w (b) on the Relative Biofilm Formation Index (RBFI), predicted by the cardinal parameter model (equation (5)).

anionic O-antigen capsule and fatty acids (Smirnova et al., 2010; Steenackers et al., 2012). Among these components, curli, fimbriae and cellulose are the fundamental elements of the extracellular polymeric matrix of *Salmonella* biofilms which in conjunction result in the formation of a highly hydrophobic network of firmly packed cells (Gerstel and Römling, 2001, 2003; Smirnova et al., 2010; Steenackers et al., 2012). Due to its complexity, the quantification of biofilm formation is not an easy task. The crystal violet assay employed in this study is a frequently used colorimetric method based on the measurement of the optical density of biofilm components in microtiter plate wells after crystal violet staining (Agarwal et al., 2011; Díez-García et al., 2012; Lianou and Koutsoumanis, 2012; Naves et al., 2008; Stepanović et al., 2000, 2003, 2004). The output of this method is a ΔOD value for the difference between the optical density measurements of the test and negative control (uninoculated) samples which provides an indirect measurement of biofilm formation.

In the present study, the RBFI was introduced as a more effective measure for the quantification of the effect of the environment on the extent of biofilm formation. The RBFI is the ratio of the ΔOD value derived from the crystal violet assay at a certain environmental condition and the maximum ΔOD value observed at the optimum condition for biofilm formation (equation (4)). As a result, the RBFI takes values from 0 (no biofilm formation) to 1 (maximum biofilm formation). For example, an RBFI = 0.5 at a certain environmental condition means that the biofilm formation (expressed in ΔOD) is 50% of the maximum biofilm that the bacterium can form at the optimum conditions. The RBFI for *S.* Newport can be predicted by the CPM developed in this study (equation (5)). Fig. 4a and b presents the combined effect of pH and a_w on the RBFI. The developed model can be used to evaluate the extent of biofilm formation in a food production line based on the intrinsic properties (pH and a_w) of the products. For example, pH and a_w combinations of 5.0 and 0.926 or 4.0 and 0.943, respectively, lead to 90% reduction (RBFI = 0.1) of biofilm formation compared to the maximum biofilm that *S.* Newport can form at optimum conditions (i.e. pH = 6.02, a_w = 0.994; Table 1). This information can be used by the food industry to optimize biofilm control by selecting, for example, the appropriate sanitation procedures.

3.2. Modelling biofilm formation boundaries

An increased number of probabilistic models predicting microbial growth/no growth boundaries have been developed until now in both liquid and solid substrates and at various environmental conditions such as pH, temperature and a_w (Koutsoumanis and Sofos, 2004, 2005; Koutsoumanis et al., 2004; Lanciotti et al., 2001; Presser et al., 1998; Valero et al., 2010; Yoon et al., 2012). Nonetheless, no attempt has been made so far, to our knowledge, towards the development of mathematical models for the description of the biofilm formation interfaces of bacterial pathogens. In the second part of this study, a probabilistic model was developed predicting the pH and a_w boundaries of *S.* Newport biofilm formation. For this purpose, the collected optical density measurements (ΔOD values), corresponding to different environmental conditions, were converted into binary data (0: no biofilm formation, 1: biofilm formation), using a threshold RBFI value equal to 0.1 (i.e. 10% of maximum ΔOD observed at optimum conditions). Based on the above critical value, among the 108 combination treatments of pH and a_w tested in the present study, biofilm formation of *S.* Newport was observed in 58 conditions and no biofilm formation in 45; there were five conditions in which biofilm formation occurred in some, but not all, of the five conducted replicates (Fig. 5).

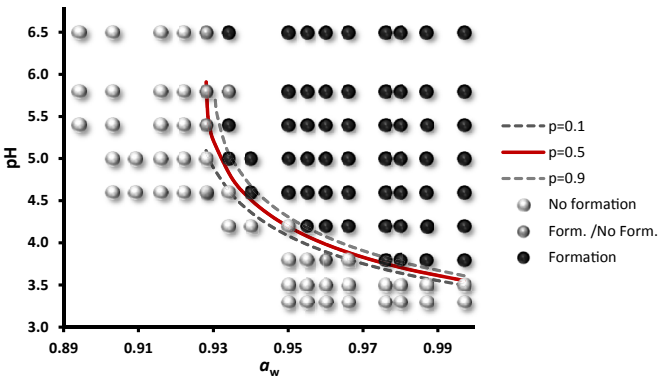

Fig. 5. Biofilm formation boundaries of *Salmonella enterica* ser. Newport at 37 °C with respect to pH and a_w predicted by the model (equation (6)) compared with the data used to generate the model. (Black symbols: biofilm formation in all replicates; grey symbols: biofilm formation observed only in some replicates; white symbols: no biofilm formation in all replicates; lower dashed line: P = 0.1; interim solid line: P = 0.5; upper dashed line: P = 0.9).

Table 2
Parameter estimates of the logistic regression model for the boundaries of biofilm formation.

Coefficients	Estimate	Standard error	*P* value
Constant	551.2	212.8	0.010
pH	−226.10	60.26	0.000
a_w	−741.5	222.9	0.001
pH x a_w	294.24	65.82	0.000
pH^2	−4.028	1.106	0.000

The binary data were further fitted to a polynomial model using logistic regression. The estimated parameters of the logistic regression model are shown in Table 2. The parameters with no significant effect ($P \geq 0.05$) were removed from the model. The concordance index and the Hosmer–Lemeshow goodness-of-fit statistic were used as measures of the goodness-of-fit of the developed model. As demonstrated by the concordance index, the degree of agreement between the predicted probabilities and the observations was 99.8%. The Hosmer–Lemeshow goodness-of-fit statistic was 6.661 (χ^2, df 8; $P = 0.574$). The goodness-of-fit was also evaluated by comparing the model predictions at probabilities of 0.1, 0.5 and 0.9 with the corresponding observed data from which the predictions were derived. As illustrated in Fig. 5, where the biofilm formation boundaries of *S.* Newport are presented, only one condition fell outside the prediction probability range (from 0.1 to 0.9).

The pH and a_w biofilm formation boundaries of *S.* Newport observed in this study are significantly wider compared to the growth boundaries of salmonellae reported in previous studies (Koutsoumanis et al., 2004; Lanciotti et al., 2001). For example, when comparing the biofilm formation boundaries observed in the present study with the growth/no growth boundaries of a *S. enterica* ser. Typhimurium strain reported in a previous study undertaken by Koutsoumanis et al. (2004), it becomes evident that the range of pH and a_w values allowing biofilm formation is much wider than that allowing growth of the pathogen: at $a_w = 0.950$, the 50% pH limit for biofilm formation is 4.19, while the respective limit for growth was estimated to be 5.12. The above data indicate that bacterial growth is not a prerequisite for biofilm formation. This is in agreement with the findings of other studies with reference to *S. enterica* strains, according to which, the growth kinetics of the tested strains did not appear to be related to their biofilm-forming ability (Díez-García et al., 2012; Lianou and Koutsoumanis, 2012). The discrete character of these two modes of bacterial growth (i.e.

planktonic and biofilm) is most likely related to the multifactoriality of biofilm formation. For instance, the conditions favoring bacterial cell adherence to a surface and/or the production of extracellular polymeric substances are not necessarily the same with those favoring planktonic bacterial growth. Moreover, the environmental conditions to which cells are exposed within a biofilm may not be the same for all cells in the biofilm (depending on their distribution and exact position in the biofilm matrix), and can be considerably different from those experienced by free cells growing in a liquid medium. All these, however, are only speculations that future research needs to ascertain, providing information of major significance for the food industry.

In conclusion, the results of the present study demonstrated that the effect of the environment on biofilm formation can be quantitatively expressed using mathematical models. The models developed in this work, although based on simplified growth media and referring to indirect (optical density) biofilm formation measurements, constitute a good basis towards the quantification of the overall interactions of biofilm growth factors and can, thus, provide useful information for controlling biofilms in food industry environments. Further research objectives of great value for the advancement of biofilm modelling approaches include the development of direct methods for biofilm quantification, the assessment and verification of the predictive power of models based on laboratory media in food substrates and food-related environments (food contact surfaces), the description of the effect of additional environmental factors (e.g., temperature, antimicrobials etc.), and the incorporation of strain variability in biofilm formation models.

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