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Modeling the Effect of pH, Water Activity, and Ethanol Concentration on Biofilm Formation of *Staphylococcus aureus*

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Abstract

In this work, the effect of environmental factors on *Staphylococcus aureus* (ATCC 13150) biofilm formation in tryptic soy broth was investigated under different ranges of pH (3.0–9.5), ethanol concentration (EtOH 0.0–20.0%), and a_w (NaCl, 0.866–0.992). Biofilm formation was quantified using the crystal violet staining method and optical density (OD: 590 nm) measurements. Biofilm formation was significantly stronger at pH and a_w close to *S. aureus* optimal growth conditions, while it was high at EtOH around 2.5–3.5 %. Data sets from the difference between the OD measurements of the test and control (ΔOD) were fitted to the cardinal parameter model (CPM) and cardinal parameter model with inflection (CPMI) to describe the effect of the environmental factors. The models showed a good quality of fit for the experimental data in terms of calculated RMSE, with the latter ranging from 0.276 to 0.455. CPM gave a good quality of fit compared to CPMI for the environmental factors tested. The optimal pH was close to neutral (6.76–6.81) and biofilm formation was possible till pH = 3.81–3.78 for CPM and CPMI, respectively. Optimum EtOH and a_w conditions for biofilm formation were in the range of 1.99–2.75 and 0.98–0.97, respectively. Predicted OD values observed using strain 13150 were very closely correlated to the OD values predicted with strain 12600 with R^2 of 0.978, 0.991, and 0.947 for pH, EtOH, and a_w, respectively. The cultivable bacterial cells within the biofilm were enumerated using standard plate counting and a linear model was applied to correlate the attached biofilm cells to ΔOD of biofilm formation. It was found that the biofilm formation was correlated with *S. aureus* population growth. At 2.5–3.5% of EtOH the maximum population density was lower than that observed at 0.0% of EtOH. As 2.5–3.5% of EtOH initiated a stronger biofilm formation, biofilm formation seems to be induced by ethanol stress. The development of cardinal parameter models to describe the effect environmental factors of importance to biofilm formation, offers a promising predictive microbiology approach to decrypting the *S. aureus* population growth and survival ability on food processing surfaces.

Keywords: *Staphylococcus aureus*, biofilm formation, pH, Water activity, Ethanol concentration, Predictive microbiology.

1. Introduction

In natural and human-made ecosystems some bacteria have the ability to attach to surfaces and form organized communities called biofilm. Its formation on food processing equipment leads to the contamination of food products, which causes foodborne illnesses and significant economic losses (Rode et al., 2007; Sharma and Anand, 2002). The microbial biofilm is a complex three-dimensional structure composed of microbial cells and an extracellular matrix principally consisting of polysaccharides, proteins, nucleic acids and lipids of microbial origin (Flemming and Wingender, 2010). These polysaccharides provide the structural scaffold of the biofilm and act as a shield against various types of antimicrobials treatments (Arciola et al., 2012; Bridier et al., 2015). It has been reported that contaminated surfaces play a pivotal role in spreading foodborne pathogens to food by contact with food processing equipment and it is one of the main contributing factors to foodborne outbreaks (Gormley et al., 2011; Perez-Rodriguez et al., 2013). It seems that biofilm formation is essential for the bacterial survival on the food processing surface and poses a potential risk of post-processing food contamination (Møretrø et al., 2003).

Staphylococcus aureus has the potential to colonize the surfaces of food processing equipment and form biofilms. *S. aureus* has often been isolated from biofilms that developed in food processing plant such as dairy, egg, seafood, and meat processing industries (Bridier et al., 2015; Gutiérrez et al., 2012; Rohde et al., 2007; Shi and Zhu, 2009; Tango et al., 2015). The survival of the *S. aureus* in hostile environments such as food processing equipment may be due to the biofilm formation, which enhances the recurrence of planktonic bacterial populations when foods are processed. Biofilms defy most antimicrobial agents and represent a potential source of bacterial contamination in the food industry. It has been reported that *S. aureus* produces polysaccharide intercellular adhesion (PIA) surrounding the cell, which can form a capsule and protects the bacterial cell against host immune response (phagocytes) (Fox et al., 2005). Previous research has shown the existence of a strong causal connection between staphylococcal biofilms and staphylococcal food poisoning (SFP) through the consumption of staphylococcal enterotoxin (SE) produced in food (da Silva Meira et al., 2012; Planchon et al., 2006). It is therefore essential for the food industry to understand the conditions, under which *S. aureus* is able to survive, grow, and contaminate food

products with respect to biofilm formation. This knowledge is critical for a successful risk assessment program.

Biofilm formation is a multistep process, involving a large number of physiological changes in bacteria and takes place in response to environmental and biochemical factors (Arrizubieta et al., 2004). The mechanism of staphylococcal biofilm formation in food industry and on medical materials has been evaluated in detail and it is reported that *S. aureus* cells form biofilms though various means. This ability to form a biofilm can be thwarted by the suboptimal growth temperature and the presence of nitrite (Gustafson et al., 2014; Rode et al., 2007). However, sodium chloride induces biofilm formation of *S. aureus* (Planchon et al., 2006). It has been reported that alcohol can induce haemolytic properties in otherwise non-haemolytic microorganisms, a phenomenon referred to as "microbial alcohol-conferred haemolysis" (MACH) (Korem et al., 2010; Knobloch et al., 2001). Korem et al. (2010) demonstrated that alcohols selectively increased the hemolytic properties of certain staphylococci strains and resulted in an increased biofilm formation. Ethanol is commonly used as plant disinfectant in food industry, medical applications, and household products, and hence may induce MACH on certain strains of *S. aureus*, thereby increasing biofilm production when used at inappropriate concentration.

The transition from a planktonic to a complex three-dimensional structure is a dynamic process that involves environmental and biochemical phenomena, thus is possibly implemented through a developmental model (Hermanowicz, 2001; Monds and O'Toole, 2009). Considerable effort has been employed during recent decades to develop mathematical models for describing substrate use and microbial population dynamics during biofilm formation. Developed models, such as individual-based models, successfully predict biofilm structure dynamics and clarify the processes which govern biofilm formation and development (Bridier et al., 2015). The development of new approaches of predictive microbiology may contribute to understanding the role of different environmental conditions on biofilm development (Hermanowicz, 2001; Xavier et al., 2004). The experimental methods will ultimately quantify the biofilm formation and mathematical models can be useful tools for investigating the effects of different environmental factors on biofilm formation by assumptions in which the enhancement or inhibitory effect of these factor is multiplicative (Ross and Dalgaard, 2003). Regarding these aspects, this study was performed with the objective of

evaluating the *S. aureus* response to pH, ethanol concentration (EtOH), and water activity (a_w) during switching between planktonic and biofilm modes and to develop a predictive microbiology model to describe the effects of these environment factors on the biofilm formation ability of *S. aureus*.

2. Materials and Methods

2.1. Bacterial Strains for biofilm testing

The modeling of biofilm development was performed using *S. aureus* strain ATCC 13150 and reference strain ATCC 12600 isolated from pork product and food processing environments. Both strains were provided by the Department of Food Science and Biotechnology, Kangwon National University, South Korea. The bacterial stock was maintained by cryopreservation at -80°C in tryptic soy broth (TSB, Difco, Sparks, MD, USA) supplemented with 15% glycerol (Sigma-Aldrich, Co, Saint-Louis, USA). Two days before each biofilm experiments, bacterial stocks were thawed and recovered from deep freezing by spreading on Bard Park Agar (BPA, difco) and incubated for 24 h at 37°C. Thereafter, single colonies were transferred in Brain Heart Infusion (BHI, difco) broth and the culture was incubated overnight during a period of approximately 18 h at 37°C, after which the overnight grown culture was used for the further biofilm development study.

2.2. Experimental conditions

The biofilm formation was studied in TSB (containing 0.5 % NaCl w/v) during a 48 h incubation period at 37°C under different combination of a_w, pH, and ethanol concentrations. The effect of NaCl concentrations was evaluated at concentrations of 2.5, 7.0, 10.0, 12.0, 14.0, 15.5, 17.0, 18.5, and 20.0 % (w/v) which correspond to a_w values of 0.983, 0.974, 0.961, 0.947, 0.934, 0.928, 0.911, 0.897, 0.887 and 0.861, respectively. TSB contained 0.5% of NaCl corresponded to a_w value of 0.992. TSB a_w was determined with a_w-meter (Aquaspector AQS-2-TC, NAGY Messysteme, Gäufelden, Germany) after adding NaCl to broth medium. The effect of pH was studied in the range of 3.0 to 9.2 in step of 0.5 and adjusted in the broth medium using hydrochloric acid (HCl 37%, Sigma, USA) or sodium hydroxide (NaOH, sigma, USA) with a digital pH meter with an epoxy refillable pH probe (Thermo Electron Corporation, Beverly, MA, USA). Previous

175 studies have demonstrated that media supplemented with low concentrations of alcohols can
176 enhance staphylococcal biofilm formation (Korem et al., 2010; Knobloch et al, 2001). Therefore,
177 ethyl alcohol (Sigma, USA) was added to the TSB to produce final concentrations of 0.0, 1.0, 2.0,
178 3.5, 5.0, 7.0, 8.5, 10.0, 12.5, 15.0, and 20.0 % (v/v). The pH, EtOH, and a_w values in TSB were
179 measured before and after autoclaving to ensure that the abovementioned values were not
180 significantly changed. Before the biofilm experiments, the *S. aureus* ATCC 13150 overnight culture
181 was adjusted to an OD of 0.05 and 2.00 at 600 nm (Ependorff Biospectrometer fluorescence,
182 Hambourg, Germany) to obtain a concentration of approximatively 5.0 and 8.0 log CFU/mL. This
183 inoculum level was confirmed by plating on TSA at 37°C for 24 h. Adjusted OD_{600} of 2.00 was used
184 as initial inoculum for modelling the extent of biofilm formation and OD_{600} of 0.05 for modeling
185 the relationship between biofilm formation and attached biofilm cells.
186
187 **2.3. Quantitative crystal violet biofilm assays**
188 Biofilm formation was evaluated using a colorimetric method which is based on the
189 measurement of the optical density (OD) of biofilms developed in polystyrene microtiter plate wells
190 (SPL Life Sciences, Pocheon, Korea) after crystal violet staining (Borucki et al., 2003; Djordjevic
191 et al., 2002). The OD_{600} of 2.00 adjusted overnight culture was diluted (1:100) into TSB containing
192 the target conditions to be tested. A total of 200 µL of diluted TSB was distributed in a 96-well
193 plate. The plates were wrapped with parafilm and incubated for 48 h at 37°C, while TSB that was
194 not inoculated was also dispensed in wells and incubated (as a blank). Subsequently, the content of
195 the plate wells was discarded (removal of non-adherent or reversibly attached cells) and the wells
196 were gently washed twice with 225 µL autoclaved phosphate buffered saline solution (PBS, Sigma).
197 The plate wells were then emptied, air-dried and stained with 225 µL of 0.1% crystal violet (Difco
198 Laboratory, Detroit, USA) for 30 min. After rinsing off the excess stain with PBS twice and air-
199 drying of the microtiter plates, the crystal violet that was bound to the formed biofilms was dissolved
200 in 230 µL of 90% ethanol (Sigma) for 30 min, and the OD was measured at 590 nm using an
201 absorbance reader spectramax i3 (Molecular device, Sunnyvale, California, USA). The
202 quantification of biofilm formation was based on the difference between the OD measurements of

the test and blank plate wells (ΔOD). Reported data are based on five independent experiments carried out with triplicate samples.

2.4. Biofilm formation and sessile cells evaluation

For attached biofilm cell enumeration, an overnight culture was adjusted to an OD of 0.05 at 600 nm to get approximatively 5.0 log CFU/mL. The adjusted 24 h culture was transferred into TSB (with 5 target conditions for each environmental parameter) as described above. Inoculated TSB was dispensed in 96-well plate wells and incubated at 37°C for 6, 12, 18, 24, 30, 36, 42, and 48 h. The biofilm quantification was performed as described above and the number of cultivable sessile cells was determined using standard plate counting as described by Hussain and Oh (2017). Broth medium in the well was discarded and the plates were gently washed four times using 225 µL of PBS to remove unbound cells. Well plates were swabbed to detach the biofilm cells and the cell suspensions were vigorously pipetted to separate biofilm cells from the swab in order to obtain single cells. Serial dilutions were prepared in PBS and 0.1 mL of the appropriate dilution was plated on TSA. Plates were enumerated after an incubation at 37 ± 2 °C for 24 h. Biofilm and cell counting evaluations were performed in two replicate experiments on separate days for each condition tested.

2.5. Model development

2.5.1. Modelling biofilm development

To describe the individual effect of NaCl, pH, and ethanol on the *S. aureus* biofilm formation, observed ΔOD values at different conditions of each factor were fitted to the cardinal parameter models (CPM) and CPM with inflection (CPMI) to determine ρ(X) for pH, a_w, and ethanol concentrations according to equation (2) proposed by Rosso et al. (1995):

$$\Delta OD = \Delta OD_{opt} \,.\, \rho(X) \quad (1)$$

$X \leq X_{min}$:

0

$X_{min} \leq X \leq X_{max}$:

$$\rho(X) = \begin{cases} \frac{(X - X_{max}) \cdot (X - X_{min})^n \cdot}{(X_{opt} - X_{min})^{n-1} \cdot [(X_{opt} - X_{min}) \cdot (X - X_{opt}) - (X_{opt} - X_{max}) \cdot (X_{opt} + X_{min} - nX)]} \\ X \geq X_{max}: \\ 0 \end{cases} \quad (2)$$

In equation (2) X_{min} and X_{max} are defined as the range of environment conditions where biofilm formation is possible, X_{opt} is the environmental condition where the optimum biofilm formation is observed, and *n* a curvature parameter (*n*=1, CPM and *n*=2, CPMI). Matlab version 9.2 R2017a (The Mathworks Inc. Natick, MA, USA) was used to determine the parameter estimates of the CPM and CPMI and for curve fitting (regression by the non-linear least squares method and the Trust-region algorithm). The calculation of the confidence bounds was based on the same equations as reported by Akkermans et al. (2017). A multi-start procedure was implemented to prevent finding a local optimum solution (100 iterations).

The developed models were evaluated using the goodness-of-fit statistic root mean square error (RMSE). The performance of developed model was assessed by graphical comparison between observed and predicted values and predictive performance factors (accuracy and bias factors).

2.5.2. Modeling the relationship between biofilm formation and attached biofilm cells

To describe the evolution of *S. aureus* populations in TSB during biofilm formation, the commonly used growth model of Baranyi and Roberts (1994) was implemented. The maximum growth rate (r_{max}, log cfu/well/h), lag phase (λ, h) and maximum population density N_{max} (log cfu/well) were estimated from the observed data at different conditions of pH, ethanol concentrations, and a_w using the DMFit version 2.1 Excel add-in software (Institute of Food Research, Norwich, England). Adjusted coefficient of determination (R^2_{adj}) and standard errors of the prediction (S_{xy}) were determined to assess the goodness-of-fit of the model. The re-parameterized Baranyi model, as the primary model, is described by the following equations:

$$N(t) = N_o + r_{max}A(t) - \ln\left[1 + \frac{e^{r_{max}A(t)} - 1}{e^{(N_{max} - N_0)}}\right] \quad (3)$$

$$A(t) = t + \frac{1}{r_{max}} + \ln\left(\frac{e^{r_{max}A(t)} + q_o}{1+q_o}\right) \quad (4)$$

$$\lambda = \frac{\ln\left(1+\frac{1}{q_o}\right)}{r_{max}} \quad (5)$$

With N(t) the cell concentration (log cfu/well) for given time t (h), N_o the initial and N_{max} the maximum cell concentrations (log cfu/well), respectively, r_{max} the maximum growth rate (1/h). A(t) is an adjustment function described by Baranyi and Roberts (1994) and can be considered as a rescaling of time, q_o is the parameter expressing the physiological state of cell when $t=t_o$ (Eq. 4), and λ is the lag time (h) (Eq. 5).

The cell concentration data of *S. aureus* in plate wells were fitted to a model proposed by Castelijn et al. (2012) to evaluate the relation between the biofilm formation ability, as assessed using the CV assay, and the concentration of cells attached on the wells' surface. The re-parameterized model is described by the following equations:

$$A_{590} = a\,.N + b \quad (6)$$

Were A_{595} is the absorbance of the solubilized CV after biofilm formation, N is the number of viable attached biofilm cells (CFU/well), *b* is the background signal (three times the standard deviation (SD) above the mean A_{595} of the negative control) and *a* is the proportionality constant between CV staining and cell counts. The parameters *a* and *b* were estimated by fitting Equation 6 to the data using GraphPad Prism version 6.03 for windows (GraphPad Software, San Diego California USA). The obtained parameters (*a* and *b*) were confirmed in Microsoft Excel by using the Excel Solver add-in. The difference between kinetic growth parameters was analyzed through an analysis of variance (ANOVA). Post-hoc tests (Tukey multiple range test) were used to determine the statistical significance of differences between growth parameters ($p< 0.05$). All statistical analyses were performed using SPSS statistics software version 22 (SPSS Inc., IBM Company, USA).

3. Results

3.1. Modelling *S. aureus* biofilm development

Initially, 25 strains isolated from different food products and plants were evaluated to select the highest biofilm producer. The evaluation was performed in TSB at 37°C for 48 h in polystyrene microtiter plates. Among these strains, ATCC 13150 produced the strongest biofilm (data no shown). *S. aureus* 13150 was studied in more detail as a target strain while investigating the effect of pH, EtOH, and a_w on the biofilm formation in TSB at 37°C after 48 h of incubation in 96-well microtiter plates. The biofilm formation was significantly stronger when pH and a_w were near the *S. aureus* growth optimal conditions. While the biofilm formation was high at EtOH around 2.5–3.5 %. Two predictive microbiology cardinal parameters models were used to describe the effect of pH, EtOH, and a_w on *S. aureus* biofilm development. Experimental data were collected from 225 biofilm formation trials to model the influence of pH on biofilm formation of *S. aureus*. The pH effect was evaluated at a_w of 0.992 and 0.0 % of EtOH concentration. The effect of pH on the biofilm formation ability of strain 13150 is shown in Fig. 1A. The results demonstrated that *S. aureus* biofilm formation depended strongly on pH variations. A very weak biofilm formation was observed at pH values lower than 4.0 and higher than 9.0. Parameter estimates and 95% confidence intervals of CPM and CPMI for pH are presented in Table 1. There was no difference between the estimate parameters from CPM and CPMI for pH. The slight difference of RMSE observed between CPM and CPMI showed that there is no inflection at suboptimal pH.

To model the effect of EtOH on biofilm formation, 155 experiments were performed at a_w of 0.992 and pH of 7.0. The biofilm formation was highest at an EtOH concentration of 3.5. The two model structures CPM and CPMI were also used to describe this effect and the fitted models are graphically shown in Fig. 2A and 2B, respectively. The RMSE value was lower in CPMI (0.276) than that observed in CPM (0.327). This illustrated that the quality of fit is greatly improved by including the inflection for high EtOH concentrations. The estimated parameters were higher in CPMI compared to those found in CPM for EtOH, except for the parameter $Eth_{min.}$ However, there is no true minimum ethanol concentration for biofilm formation and consequently the parameter Eth_{min} is given a theoretical negative value. Consequently, this parameter has no interpretation.

To model the effect of a_w using NaCl concentration on the biofilm formation, 155 experiments were performed at a pH of 7.0 and 0.0% of EtOH. Both curvature parameter n=1 and n=2 were performed to describe the effect of this factor and the fitted models are graphically displayed in Fig. 3A and 3B, respectively. The estimate parameters for CPM were almost equal to those observed for CPMI for a_w. RMSE value was lower in CPM (0.276) compared to that calculated in CPMI (0.327), demonstrating the better quality of fitting of the former compared to the latter model.

3.2. Validation

The validation of cardinal models was performed using a goodness of fit statistics and prediction accuracy indices. Firstly, to obtain a measure for the agreement between the experimental data and the developed cardinal models, the experimental data were plotted against predicted data using a linear regression. The average coefficient of determination (R^2) values were 0.918 for CPM and 0.86 for CPMI, suggesting that the correlation between experimental data and predicted data were better for CPM than CPMI for all factors used in the present study (Fig. 4).The accuracy factor A_f and bias factor B_f were also calculated for both CPM and CPMI and each influencing factor. The results of A_f and B_f are summarized in Table 1. The cardinal models performed well for these datasets with average A_f of 1.107 and 0.996 and B_f of 1.273 and 1.320, respectively for CPM and CPMI. In general, the accuracy and bias factors were acceptable as described by (Ross, 1999).

3.3. Modeling the correlation between planktonic cells and biofilm formation

To investigate the growth of biofilm attached populations during biofilm formation, 5 conditions for each factor (pH, EtOH, and a_w) were selected among the described conditions used to study biofilm formation. The Baranyi and Roberts (1994) model was fitted to the population counts and growth parameters were presented in Table 2. To model the correlation between the biofilm attached *S. aureus* population and biofilm formation, the datasets were fitted to a linear model to determine the factor *a*. The factor *a* represents a proportionality constant between the attached *S. aureus* biofilm populations and OD observed. The linear model was graphically shown in Fig. 5 and factor *a* was presented in Table 2. The results showed that the factor *a* from the linear

model was higher in TSB at pH 7.0 (a= $2.85x10^{-6}$) compared to those observed in TSB at pH 4.0 and 9.5 (a=$7.88x10^{-8}$ and $6.59.10^{-8}$). The growth parameters r_{max} and N_{max} were higher in TSB with pH 7.0 compared to those found for pH 4.0 and 9.5. Similar results were observed when biofilm formation was studied in TSB supplemented with different concentrations of NaCl. The factor *a* was higher at the optimal growth condition than suboptimal growth conditions. These results suggest that the biofilm formation is correlated with *S. aureus* population growth (Castelijn et al., 2012; Kadam et al., 2013). For EtOH, the results showed that N_{max} (9.45 log cfu/well) was higher at TSB without ethanol compared to those found in TSB supplemented with 3.5 and 7.0 % of ethanol (8.84 and 8.91, respectively). Moreover, the factor *a* was higher (a = $3.55.10^{-6}$ and $2.91.10^{-6}$) for the developed model for results in TSB supplemented with 3.5 and 7.0 %, respectively, compared to that found in TSB with 0.0 % of ethanol. These results indicate that the extracellular matrix production did not only depend on the *S. aureus* population growth but also on stress induces by ethanol at the non-lethal concentration.

4. Discussion

S. aureus has been recognized as one of the greater biofilm producer bacteria and the connection between staphylococcal biofilm and staphylococcal food poisoning has been previously established (da Silva Meira et al., 2012; Planchon et al., 2006). In the present study, the effect of environmental parameters on *S. aureus* biofilm formation was modeled using CPM and CPMI for pH, EtOH, and a_w. The cardinal parameters are a family of models which define cardinal values (minimum, optimum and maximum) of environment factors (temperature, pH, aw, organic acids …) from the microbial growth rates observed at optimal or non-optimal conditions for other environmental factors (Rosso et al., 1995). The CPMI was designed first to describe the effect of temperature on microbial growth rate (Rosso et al., 1993). CPM was later extended to other environmental factor including pH and aw (Rosso et al.; 1995; Rosso and Robinson, 2001). These models are commonly used to describe and to predict the effect of environment factors on microbial growth because they are based on parameters that are biologically meaningful and have no structural correlation. Recently the cardinal pH and a_w models were successfully used to fit on experimental data of *Salmonella enterica* ser. Newport biofilm formation in TSB (Dimakopoulou-Papazoglou et

al., 2016). To our knowledge, no data are available on the effects of environmental factors on *S. aureus* biofilm formation. Therefore, herein both CPM and CPMI were effectively fitted to a data set of *S. aureus* strain ATCC 13150. With the average goodness-of-fit index value close to 1.0, the cardinal parameters and predicted biofilm formations were almost similar to experimentally observed biofilm formation, indicating that the predictive biofilm formation models were accurate for all condition used in the present study. Regarding goodness of fit statistic and prediction accuracy, CPM gave a better quality fit compared to CPMI for *S. aureus* biofilm formation irrespective of the environmental factors used in this study.

Individual-based modelling is an approach to understand and predict the processes that lead to the development of microbial populations (spoilages and pathogens). Currently the use of cardinal parameter models allows to describe the effects of environment factors (pH, EtOH, and aw) on the switching between planktonic and biofilm modes. The results demonstrated that the *S. aureus* produced stronger biofilms at pH and a_w values close to optimal conditions of growth. The weak biofilm formation was found within the range of pH and a_w that does not allow *S. aureus* growth. Furthermore, the high biofilm production depended closely on the population growth of *S. aureus*. Dimakopoulou-Papazoglou et al. (2016) studied the effect of pH and a_w on biofilm formation ability of *Salmonella enterica* and concluded that biofilm formation required a similar range of pH and a_w as needed for *Salmonella enterica* growth. It has been reported that the addition of NaCl in the growth medium stimulated staphylococcal biofilm formation (Knobloch et al., 2001; Lim et al., 2004; Møretrø et al., 2003). However, herein high percentages of NaCl interfered with bacterial growth and biofilm formation. For EtOH, the higher biofilm formation may be due to a stress response induced by ethyl alcohol at the non-lethal concentration. The concentration range of 2.5–3.5 % of EtOH slightly interfered with the *S. aureus* growth because N_{max} at these concentrations was lower than observed at 0.0% of EtOH. Therefore, a concentration range of 2.5–3.5 % was found as non-lethal concentrations activating a stronger biofilm formation in *S. aureus* strain 13150. A number of studies have reported that treatments with low concentrations of alcohols can enhance the formation of staphylococcal biofilm. Korem et al. (2010) studied the hemolytic effect induced by alcohols and they reported that ethanol (2.4 %) increased the expression of multiple surface proteins, which might be important for cell attachment. Redelman et al. (2012) proved that different

alcohols (ethanol, isopropanol, methanol…) induced stress which significantly affected *S. aureus* biofilm formation, partly through enhanced production of extracellular matrix and other biofilm-promoting factors (Archer et al., 2011). In the future, it will be interesting to move this research forward to include other alcohols commonly used as sanitizers in the food industry and hospitals.

In order to see whether the *S. aureus* growth is correlated with an increase in biofilm formation a combination of the Baranyi and Roberts (1994) model and a linear model described by (Castelijn et al., 2012; Kadam et al., 2013) was applied to calculate the growth parameters and the factor *a*. The results showed a clear correlation between the growth parameters and biofilm formation. Low lag time, high growth rate and maximum population density was associated with high biofilm formation irrespective of the environmental conditions used in this study. The bacterial density is of relevance as attached biofilm cells can contaminate foods during processing. The risk of *S. aureus* biofilm formation would thus greatly dependent on bacterial population density and stress conditions induced by nonlethal concentrations of ethanol. This result indicates that *S. aureus* populations survive on food processing equipment and low concentrations of ethanol remaining after disinfection may constitute a potential risk of biofilm formation under environmental stress, therefore increasing the food safety risk.

5. Conclusion

The present study developed and validated a predictive model to quantitatively assess the effects of pH, EtOH, and a_w on the biofilm formation ability of *S. aureus*. The developed cardinal models allow defining the range of environmental factors for which the biofilm formation is probable. These models were able to estimate the rate of *S. aureus* biofilm formation. Subsequently these models can play a great role in risk assessment. The models are based on simplified growth media and refer to indirect (optical density) biofilm formation measurements. Therefore, more information about biofilm development on real food matrices and industrial food relevant surfaces will permit optimization of their risk assessment.

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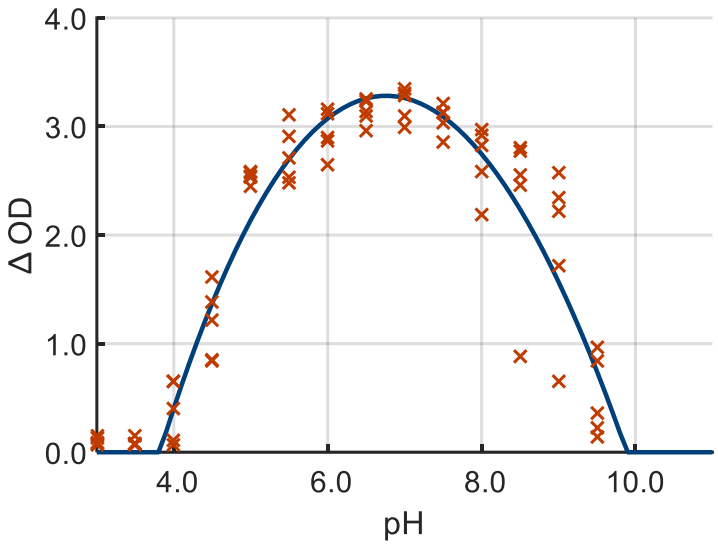
549 **FIGURES**

550 A



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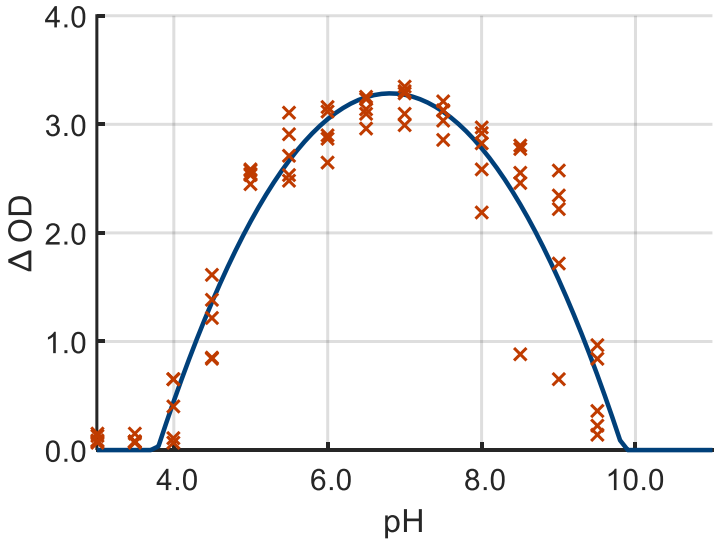


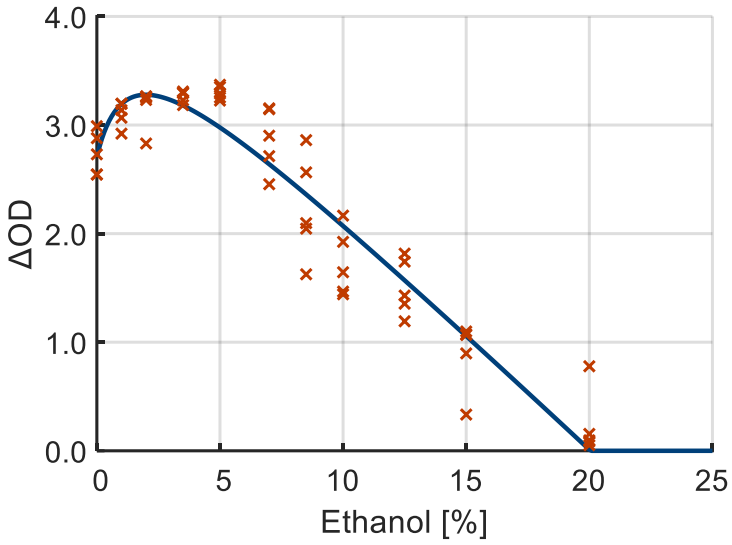
553

554 **Figure 1.** Cardinal parameter model for the effect of pH on *Staphylococcus aureus* biofilm

555 formation. A= cardinal parameter model, B= cardinal parameter model with inflection.

556

A



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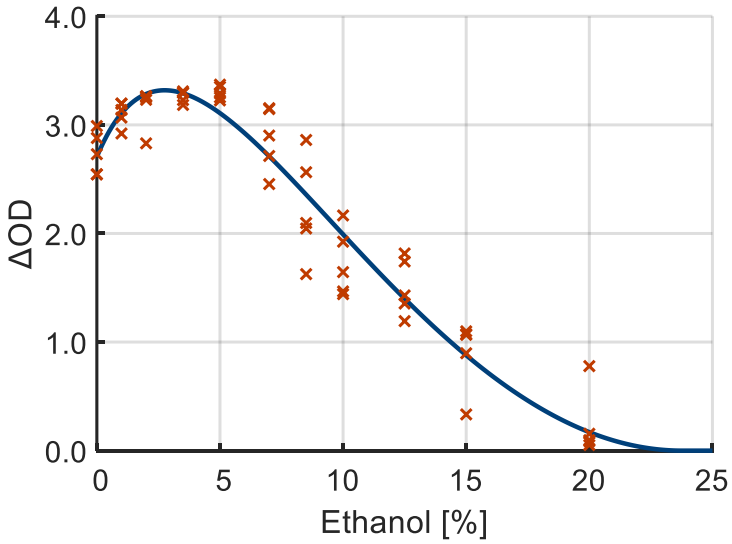

Figure 2. Cardinal parameter model for the effect of ethanol (%) on *Staphylococcus aureus* biofilm formation. A= cardinal parameter model, B= cardinal parameter model with inflection.

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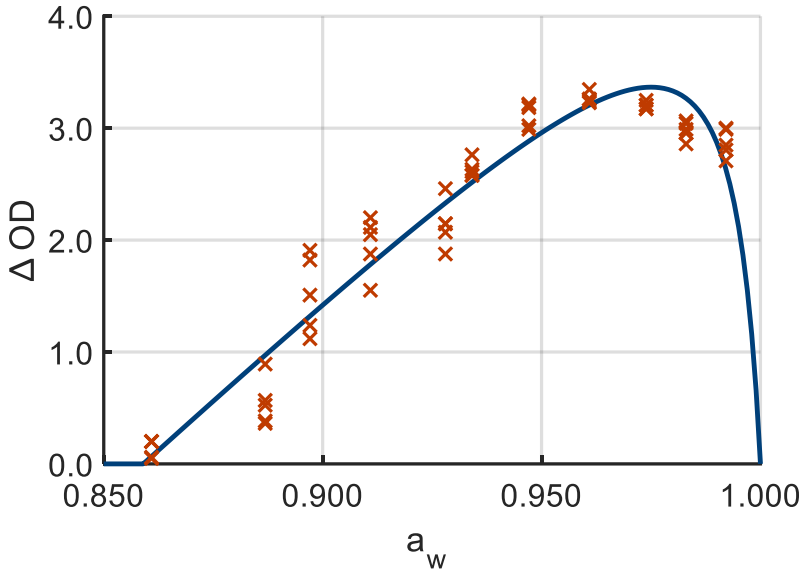

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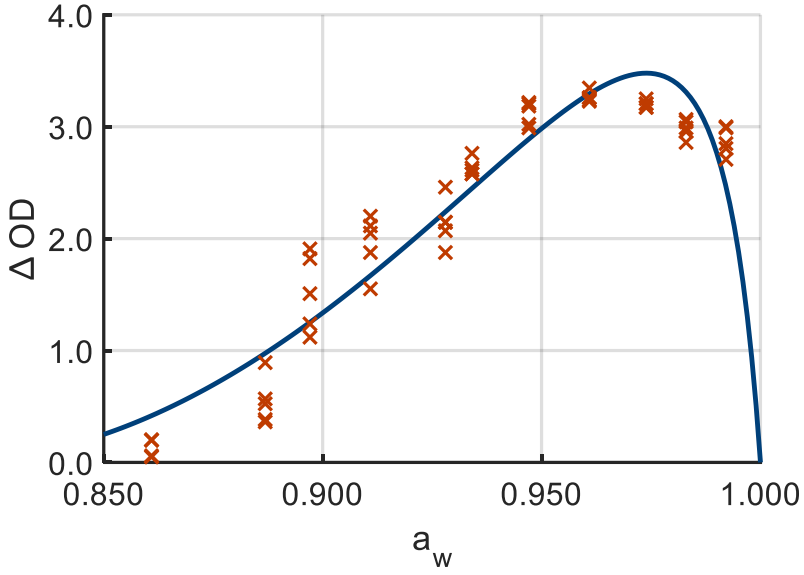
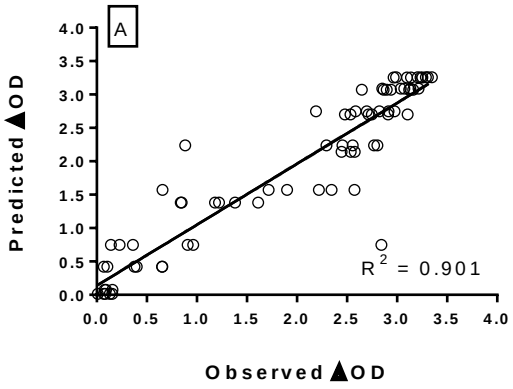


Figure 3. Cardinal parameter model for the effect of aw on *Staphylococcus aureus* biofilm formation. A= cardinal parameter model, B= cardinal parameter model with inflection.



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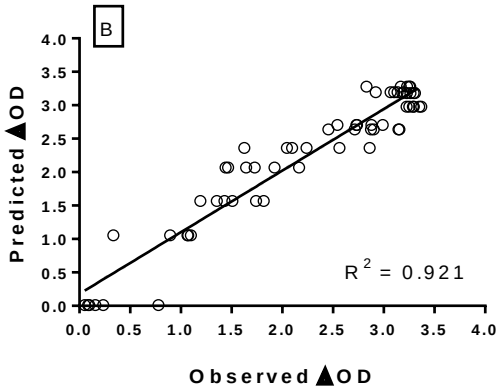

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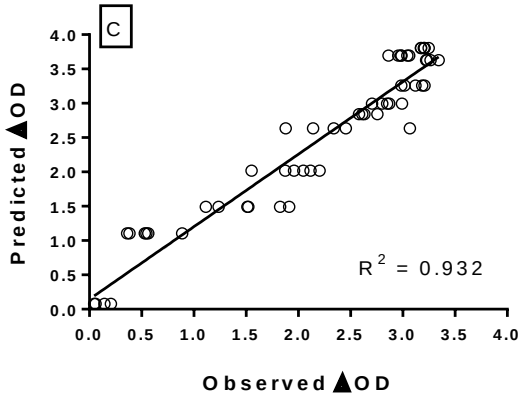

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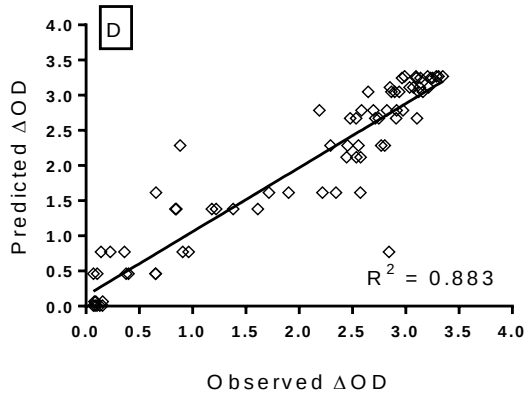

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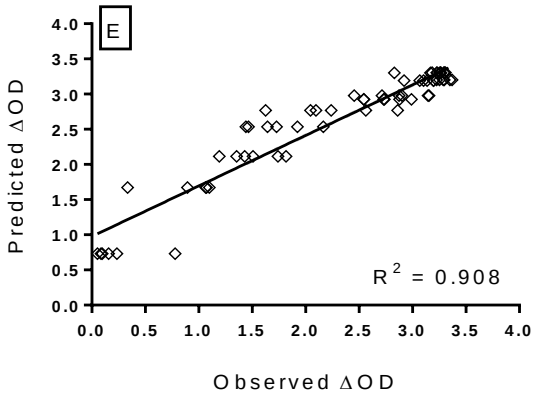

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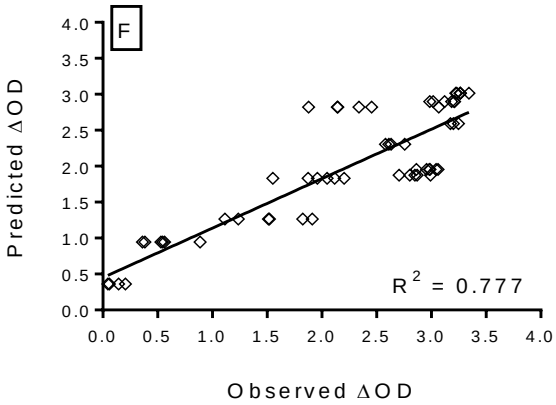

578

579 **Figure 4.** Comparison between predicted and observed ΔOD values for biofilm formation of
580 *Staphylococcus aureus* at various environmental conditions. Cardinal parameter model for pH (A),
581 cardinal parameter model for ethanol (B), cardinal parameter model for aw (C), cardinal parameter
582 model with inflection for pH (D), cardinal parameter model with inflection for ethanol (E), cardinal
583 parameter model with inflection for aw (F).

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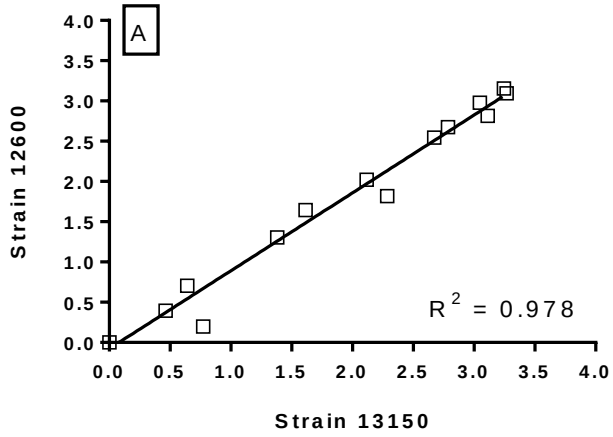

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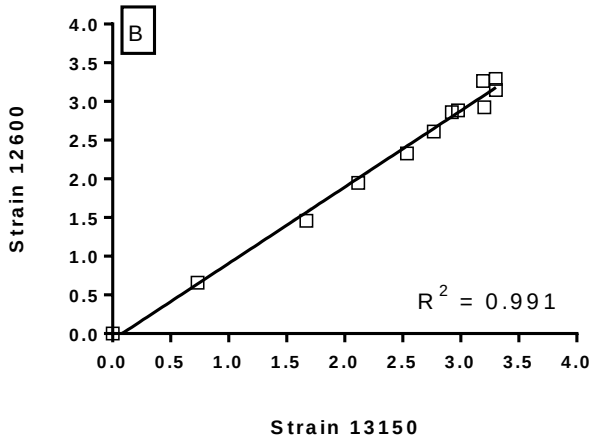

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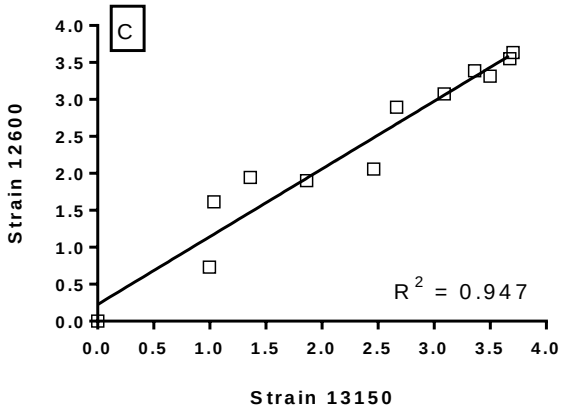

587

588 **Figure 5.** The correlation between the crystal violet staining assay and plate counts of
589 *Staphylococcus aureus*. Biofilm was formed in TSB with different pH (A), ethanol concentration
590 (B) and a_w (C) at 37 °C for 6 h–48 h. Absorbance values from the CV assay (OD590 nm) are plotted
591 against the log CFU/well and fitted with the linear equation.

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TABLES

Table 1. Parameter estimates, with 95% confidence intervals (indicated with ±) and goodness-of-fit statistic of CPM and CPMI describing the effect of pH, ethanol (% i.e. v/v), and a_w on ΔOD.

Factor	Parameter	CPM		Statistic	CPMI		Statistic
pH	pH_{min}	3.81	± 0.08	RMSE = 0.448	3.78	± 1.12	RMSE = 0.455
	pH_{opt}	6.76	± 0.10	B_f = 1.007	6.81	± 0.05	B_f = 1.222
	pH_{max}	9.89	± 0.11	A_f = 1.111	9.84	± 0.08	A_f = 1.311
	ΔOD_{opt}	3.28	± 0.07		3.28	± 0.08	
Ethanol	Eth_{min} (%)	0.93	± 0.97	RMSE = 0.317	-2.35	± 1.57	RMSE = 0.280
	Eth_{opt} (%)	1.99	± 0.67	B_f = 1.292	2.75	± 0.50	B_f = 0.771
	Eth_{max} (%)	20.05	± 1.14	A_f = 1.416	23.76	± 2.62	A_f = 1.303
	ΔOD_{opt}	3.27	± 0.17		3.32	± 0.15	
a_w	$a_{w,min}$	0.86	±0.003	RMSE = 0.276	0.81	± 0.01	RMSE = 0.327
	$a_{w,opt}$	0.98	± 0.001	B_f = 1.021	0.97	± 0.001	B_f = 0.996
	ΔOD_{opt}	3.37	± 0.06	A_f = 1.293	3.48	± 0.07	A_f = 1.344

CPM: Cardinal parameter models; CPMI: Cardinal parameter models inflection. RMSE: Root Mean Square Error; B_f: Bias factor; A_f: Accuracy factor.

598 **Table 2.** Parameter *a* estimated from equation (6) and growth parameters from Baranyi and Roberts model.

Factor	Condition	*a*	Growth parameters			R^2_{adj}
			r_{max}	λ	N_{max}	
pH	4.00	$7.88x10^{-8}$b	0.058a	14.56d	6.14b	0.989
	5.50	$1.76x10^{-6}$c	0.172b	5.15c	8.86c	0.987
	7.00	$2.85x10^{-6}$d	0.249c	2.45a	9.04d	0.986
	8.50	$1.83x10^{-6}$c	0.224c	3.67b	8.96cd	0.997
	9.50	$6.59x10^{-8}$a	0.034a	18.55a	4.94a	0.839
Ethanol (%)	0.0	$2.12x10^{-6}$c	0.284d	3.81a	9.45d	0.987
	3.5	$3.55x10^{-6}$d	0.164c	5.01b	8.84c	0.990
	7.0	$2.91x10^{-6}$e	0.155c	5.47b	8.91c	0.994
	12.5	$5.55x10^{-7}$b	0.110b	13.6c	7.88b	0.988
	20.0	$1.66x10^{-7}$a	0.041a	20.9d	6.95a	0.967
a_w	0.99	$1.75x10^{-6}$c	0.289d	3.95a	9.46d	0.997
	0.98	$2.77x10^{-6}$d	0.235c	4.08ab	9.31c	0.871
	0.96	$3.15x10^{-6}$e	0.233c	4.20b	9.15c	0.901
	0.93	$9.89x10^{-7}$b	0.164b	14.14c	7.76b	0.938
	0.88	$2.22x10^{-8}$a	0.059a	16.63d	6.48a	0.974

599 *a*: proportionality constant between CV staining and cell counts, r_{max}: maximum growth rate (1/h); λ: Lag time (hour); N_{max}: maximum population density (log

600 cfu/well). Different letters in the same column indicate significant differences ($p<0.05$) for estimated parameters.

601