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A PREDICTIVE GROWTH MODEL FOR *Staphylococcus aureus* IN COASTAL CHEESE COATED WITH ACTIVE FILM CONTAINING AQUEOUS EXTRACT OF *Gliricidia sepium*

MODELO PREDICTIVO DE CRECIMIENTO DE Staphylococcus aureus EN QUESO COSTEÑO RECUBIERTO CON FILM ACTIVO QUE CONTIENE EXTRACTO ACUOSO DE Gliricidia sepium

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RESUMEN

This study was conducted to develop a predictive model in order to analyze the effects of active film containing aqueous extract of *Gliricidia sepium* (AEG) against *Staphylococcus aureus* growth in coastal cheese. Inoculated coated cheese samples were stored at 6, 12, 18 and 21°C, and the change in the populations of *S. aureus* during storage were analyzed using Huang model to determine specific growth rate (μmax), lag-phase duration (λ), and maximum population density (Ymax). The polynomial equation was used as the secondary models to describe the effect of temperature on μmax and predict the behavior of *S. aureus.* The model performance was

evaluated by the root mean square error (RMSE), bias factor (Bf), and accuracy factor (Af). The secondary model had Af and Bf factors close to one, showing that model can describe the *S. aureus* growth in cheese covered with active film. These results highlighted that AEG incorporation in gellan gum is a promising approach to control risk of foodborne contamination in a real food system as a Colombian coastal cheese. Therefore, the developed models were acceptable for predicting the growth of *S. aureus* in coastal cheese under likely temperature abuse conditions and can be applied to assess the risk of *S. aureus* in cheese and design temperature controls to reduce the risk of staphylococcal food poisoning.

Keywords: active film, cheese, secondary model, aqueous extract, predictive microbiology.

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RESUMEN

Este estudio se realizó para desarrollar un modelo predictivo con el fin de analizar los efectos de la película activa que contiene extracto acuoso de Gliricidia sepium (AEG) contra el crecimiento de Staphylococcus aureus en queso costero. Las muestras de queso recubierto inoculado se almacenaron a 6, 12, 18 y 21 °C, y el cambio en las poblaciones de S. aureus durante el almacenamiento se analizó utilizando el modelo de Huang para determinar la tasa de crecimiento específica (μmax) y la duración de la fase de retraso (λ). y densidad de población máxima (Ymax). La ecuación polinómica se utilizó como modelo secundario para describir el efecto de la temperatura en μmax y predecir el comportamiento de S. aureus. El rendimiento del modelo se evaluó mediante el error cuadrático medio (RMSE), el factor de sesgo (Bf) y

el factor de precisión (Af). El modelo secundario tenía factores Af y Bf cercanos a uno, lo que demuestra que el modelo puede describir el crecimiento de S. aureus en queso cubierto con una película activa. Estos resultados resaltaron que la incorporación de AEG en la goma gellan es un enfoque prometedor para controlar el riesgo de contaminación transmitida por alimentos en un sistema alimentario real como el queso costero colombiano. Por lo tanto, los modelos desarrollados fueron aceptables para predecir el crecimiento de S. aureus en el queso costero en condiciones probables de abuso de temperatura y pueden aplicarse para evaluar el riesgo de S. aureus en el queso y diseñar controles de temperatura para reducir el riesgo de intoxicación alimentaria por estafilococos.

Palabras clave: película activa, queso, modelo secundario, extracto acuoso, microbiología predictiva.

INTRODUCTION

Staphylococcus aureus is one of the most common causes of reported foodborne diseases worldwide. It can grow at various ranges of pH, water activity (A_w) and temperature, leading to intoxication from an extensive range of food. Staphylococcal enterotoxins are associated with protein-rich food, such as dairy products, which might be subject to extensive manual handling, inappropriate storage or inadequate heating (Wallin-Carlquist *et al.,* 2010).

Coastal cheese is a dairy product commercialized mainly in the Colombia Caribbean region. It is manufactured by enzymatic coagulation without any thermal treatment. Its high A_w and composition encourage *S. aureus* to grow and produce enterotoxins (Lin *et al.,* 2018). The coastal cheese is subjected to temperature abuse during manufacturing, distribution, and storage hence it is susceptible to *Staphylococcus aureus* contamination. Therefore, controlling the growth of *S. aureus* is an important topic for ensuring the safety of coastal cheese products.

Consumers prefer food with good quality, long shelf life, minimum preservatives and

no synthetic additives. The demand for strategies to increase product shelf life has led to the development of active food packaging films (Valdramidis and Koutsoumanis, 2016). Studies have been carried out about developing an innovative packaging on cheese to prolong microbial shelf life (Gorrasi *et al.,* 2016).

Edible films and coatings had a remarkable interest increasing shelf life and enhancing food quality and safety, which is expected to play an important role in the food market in the future years. Moreover, these matrixes can be employed as carriers of active compounds such as antimicrobials to reduce the risk of foodborne contamination. Extending the microbial shelf life of cheese is an important topic to the dairy industry due to the high interest in extending the distribution beyond the market borders. The incorporation of natural additives could be a fascinating strategy to design a better antimicrobial packaging system; hence, the incorporation of natural components had been suggested by some researchers (Azadbakht *et al.,* 2018).

Predictive microbiology has been used to predict cheese's microbial shelf. As environmental factors influence microorganisms' growth, predictive models can be valuable for risk assessment, especially estimating changes in the bacterial population in a food chain. Moreover, microbial prediction is considered an interesting alternative to prevent food poisoning. Different statistical indices such as root mean square error (RMSE), accuracy factor (Af) and bias (Bf) have been proposed to evaluate the quality fit of one model (Geitenes *et al.,* 2013).

There are primary and secondary models widely used in predictive microbiology. Primary models describe the variations in bacterial cells as a function of time; whereas, secondary models refer to the responses of growth parameters produced by environmental conditions fluctuations. Mathematical models that describe the effect of temperature on the growth of *S. aureus* have been published (Kim *et al.,* 2018).

This study's objective was to examine how storage temperature affects the growth of *S. aureus* in coastal cheese coated with an active film made from gellan gum and aqueous extract of *S. balansae.* The second objective was to develop mathematical models that may be used to predict the growth of *S. aureus* in cheese as affected by storage temperature.

MATERIAL AND METHODS

Bacterial strains and growth conditions.

S. aureus was obtained from the food microbiology laboratory of the University of Cartagena (Colombia). 1mL of the culture was mixed into 10 mL in tryptic soy broth (TSB) incubated at 37 °C for 24 h in order to activate the inoculum. Then 1 mL of the activated strains were subjected to a second activation in TSB (37 °C, 24 h) to attain a viable bacteria population of about 2 log CFU/mL.

Steam distillation system.

The distillation process was carried out through a distillation system composed by a volumetric flask container (VFC) as a heat source to generate steam by boiling water. The VFC is linked to another spherical glass container (SGC) with two entrances, the upper entrance receives the VFC and the bottom entrance is coupled to a glass condenser. A glass collector recovers the aqueous extract from vegetal material into the condenser.

The leaves of *G. sepium* were placed in the SGC flask and water in the VFC. Finally, water is heated and the vapor passes through the leaves in the SGC dragging active principles that are condensed and recovered as an aqueous extract in the glass collector.

Gas chromatography - mass spectrometry.

50 µl of aqueous extract was added up to 450 µl with dichloromethane; subsequently, 2 mL were transferred to a vial for gas chromatography. It was used a gas chromatograph (Agilent Technologies 7890A), coupled to a mass spectrometer (Agilent Technologies 5975C) and HP Chem Station data system employing a DB-5MS capillary column (J & W Scientific, Folsom, USA) with stationary phase of 5% phenylpolymethylsiloxane (60 m x 0.25 mm, D.I. x 0.25 µm) for the separation. Oven temperature was from 45ºC (5 min) to 150ºC (2 min) at a rate of 4ºC/min.

Film preparation.

0.5 % (w/v) of low acyl gellan (LAG) was dissolved in deionized water at 80°C during 10 minutes. Then, glycerol as a plasticizer (8.0% w/v) was added to the solution and stirred for 10 min. The solution was air-conditioned in order to incorporate the aqueous extract. Air bubbles in the solution were then removed by ultrasonic treatment for 5 min. 25 mL of the film solution was casted on a petri dish and dried for 48 h at 34°C controlling the edible film thickness.

Inoculation and enumeration of *S. aureus*.

The cheese samples were purchased at a local supermarket located in Cartagena city (Colombia). Initially, the cheese samples were cut into 11 g portions and then immersed into *S. aureus* solutions with bacterial cell concentrations of 2.0–3.0 log CFU/mL for about 5 min. The inoculated samples were then packaged aseptically and stored at different temperatures (6, 12, 18 and 21°C) during various days including the blank samples (without cover).

To obtain the microbial growth curve the samples were mixed with 99 mL of peptone water and homogenized in a stomacher and each dilution was spread plated onto Baird–Parker agar (Merck, Darmstadt, Germany). Populations of *S. aureus* cells were enumerated at appropriate time intervals.

Primary modeling.

Growth curves of *S. aureus* at each temperature were constructed by plotting the logarithm of the number of microorganisms versus time. Each point of the growth curve corresponds to the mean value of the entire set of samples assessed. The growth curves obtained under each storage temperature were analyzed to determine the specific growth rate (μmax), lag phase duration (λ), and maximum growth population (Ymax) by fitting the data to Huang model (Eq 1 y Eq 2).

$$[\,Y(t) = Yo + Ymax - Ln\{\,e^{Yo} + [e^{Ymax-Yo}]e^{\mu max\,B\,(t)}\}] \qquad Eq\ 1.$$

$$\left[B(t) = t + \frac{1}{\alpha}\,Ln\,1 + \frac{e^{-\alpha(t-\lambda)}}{1+e^{\alpha\lambda}}\right] \qquad Eq\ 2.$$

Where y_0, y_{max} and $y_{(t)}$ are the bacterial concentration at initial, maximum, at time t; μ_{max} represents the maximum growth rate [(Log CFU/g)/h], and λ represents the latency phase. The latency phase coefficient is α 4.

Secondary modeling.

The growth rates (μmax) obtained from Huang model were modelled as a function of temperature employing a polynomial model (Eq 3).

$$Ln\,(x) = a + b * T + c * T^2 \qquad Eq\ 3.$$

Where, x is the growth rate. a, b, and c are constants and T is temperature.

Validation of predictive models.

The Af, Bf and RSME were used to evaluate the goodness of the generated secondary model. The equations are the following:

$$\left[Af = 10^{(\Sigma\,|\frac{\log \mu pred}{log\ \mu obs}|/n)}\right] \qquad Eq\ 4$$

$$\left[Af = 10^{(\Sigma \log (\frac{\mu obs}{\mu pred})/n)} \right] \quad Eq\ 5$$

$$RSME = \frac{\Sigma(obs - pred)^2}{n} \quad Eq\ 6.$$

where, the variable factors obs, pred, and n are the observed values, predicted values, and repetition number of the observed data, respectively.

Statistical analysis.

The results for growth parameters of *S. aureus* were reported as means ± standard deviation. The influence of temperature on maximum specific growth rate (μmax) was evaluated employing the analysis of variance (ANOVA one-way) along with Tukey test to determine statistical differences ($P < 0.05$) using SPSS software version 23.0 for Windows.

RESULTS AND DISCUSSION

Gas chromatography - mass spectrometry

Leaves of *G. sepium* cultivated in Colombia were submitted to steam distillation. GC/MS analyses performed on the aqueous extract obtained showing 10 compounds (68.68 %). Table 1 shows the main compounds identified along with retention time and the concentration area, where it can be observed that major compounds were: Ethanoioic Acid (23.19%), Mercaptoacetic acid (9.85%), 3, 4-Dihydroxybenzyl alcohol (8.45%) and 2-hydro-4-methylpentanoic acid (6.02%), 2-Ethoxybutane (4.76%), 2,6-oi-hydroxy acetophenone (4.46%), 3-animo-2-phenazinol (2.25%), 17-epi-trenbolone (2.32%), N- (Trifluoroacetyl) norepinephrine (2.35%) and N - (Trifluoroacetil) Epinephrine (2.03%).

Table 1: Chemical composition of aqueous extract of *Gliricidia sepium*.

Compounds	Retention time (min)	Concentration area (%)
2-Ethoxybutane	10.840	4.76
2- Hydro-4-methylpentanoic acid	6.333	6.02
2.6-dihydroxyacetophenone	12.736	4.46
3-amino-2-phenazinol	18.490	2.25
17-epi-trenbolone	24.454	2.32
3.4-Dihydroxybenzene alcohol	34.491	8.45
N- (Trifluoroacetyl) norepinephrine	34.530	2.35
N- (Trifluoroacetil) Epinefrina	34.552	2.03
Ethanoioic Acid	38.867	23.19
Mercaptoacetic acid	42.768	9.85

Other authors have evaluated the chemical composition and antimicrobial properties of extracts of *G. sepium* (matarratón), *B. orellana* (achiote), *J. mimosifolia* (gualanday), and *P. pulchrum* (desvanecedora) found that flavonoids are the common compounds (Mgbeahuruike *et al., 2017; A*lonso *et al., 2018*). Tajkarimi, Ibrahim & Cliver, (2010) reported that vegetables' components could depend on the geographical, region, age of the plant, harvest time, harvesting season, drying method, and storage condition and method of extraction.

Fitting Huang model.

The growth curves at 6, 12, 18 and 21°C clearly exhibited the lag, exponential growth, and stationary phases. Figure 1 shows a strong increase in the population of *S. aureus* in blank samples; while, in covered cheeses, the increase was less sharp due to the application of active films. These results could be attributed to the aqueous extract, confirming the antimicrobial effect of the compounds previously reported in aqueous extract of *G. sepium.*

Fitting the growth curves to the Huang model allows for determining growth parameters such as initial count cells (Y_0), maximum latency phase (λ), maximum growth rate (μmax) and maximum cell population (Ymax). Y_0 values were not altered significantly (P>0.05) by temperature changes or active film application (Table 2).

Table 2. Growth parameters of *S. aureus* at different temperatures using the Huang model.

Temperature (°C)	Parameters	blank sample	Cheese covered with active film
21	Y_0 (log CFU)	2.085	2.027
	λ (min)	15.98	136.111
	Y_{max} (log CFU)	7.531	4.289
	μ(min^{-1})	0.065	0.120
18	Y_0 (log CFU)	2.053	2.018
	λ (min)	26.454	139.379
	Y_{max} (log CFU)	7.515	3.935
	μ(min^{-1})	0.054	0.090
12	Y_0 (log CFU)	2.007	2.013
	λ (min)	52.345	142.324
	Y_{max} (log CFU)	7.142	3.668
	μ(min^{-1})	0.034	0.010
6	Y_0 (log CFU)	2.020	2.032
	λ (min)	93.437	153.643
	Y_{max} (log CFU)	7.237	3.572
	μ(min^{-1})	0.025	0.009

Within a row, means lacking a common lowercase letter are different (p<0.05).

Y_0 had values between 2.007 and 2.085 log CFU indicating that Y_0 can be controlled when the bacteria are incorporated into the food system. The initial number of *S. aureus* is a crucial safety indicator in artisanal dairy practice (Acai *et al.,* 2014).

λ, is the necessary time that microorganisms take to adapt to a new environmental or nutritional condition (Swinnen *et al.,* 2004). This variable showed to be dependent on the temperature and active film application. The highest value was found in cheese samples covered with active film and stored at 6°C (153.643 h^{-1}), followed by samples stored at 12 (142.324h^{-1}), 18 (139.379h^{-1}) and 21°C (136.111h^{-1}) respectively. While, cheese samples without covering had values at 6°C, λ 93.437h^{-1}; at 12°C, λ 52.34h^{-1}; at 18°C λ 26.454; at 21°C, λ 15.980.

These results suggest that λ parameter was more prolonged in samples covered with active film than blank samples. Similar results were published by Lin *et al.,* (2018), who found that λ of *S. aureus* in cooked pork sausage containing preservatives such as sodium nitrite, nisin, and potassium sorbate was longer than that samples without preservatives.

μmax is a parameter depending on the environmental conditions. Lower values were obtained in cheese samples covered with active films at 6°C, μmax 0.009 h-1; 12°C, μmax 0.010 h^{-1}; 18°C, μmax 0.090 h^{-1} and 21°C, μmax 0.120 h^{-1} in comparison with blank samples at 6°C, μmax 0.025 h-1; 12°C, μmax 0.034h-1; 18°C, μmax 0.054h^{-1} and 21°C μmax 0.065h^{1}.

These values are different from those found by Lu *et al.,* (2020) in cooked rice with pork floss. These authors evaluated *S. aureus* growth using Huang model reporting μmax values of 0.53h^{-1} at 25°C; 0.23 h^{-1} at 18°C and 0.09 h^{-1} at 12°C; that difference can be attributed to the nutritional composition of the substrate (different food). Some authors mentioned that μmax decreases are caused by limitations of nutrients and oxygen (Skandamis and Jeanson 2015). The last parameter calculated by the Huang model was Ymax, which corresponds to the maximum bacterial concentration reached at the final of the exponential phase. The importance of this parameter lies in that some microorganisms require a certain number of bacteria to produce toxins.

In cheese covered, the highest Ymax value (4.289 log CFU) was reached at 21°C followed by 18°C and 12°C with 3.935 and 3.668 log CFU respectively. Regarding to blank samples, the highest value was obtained at 21°C with 7.531 log CFU, while

the lowest was reached at 12°C (7.142 log CFU).

In general terms, the Ymax, values were higher in blank samples than cheese covered with active films. Based on this result, it could be drawn that aqueous extract of *G. sepium* could significantly inhibit *S. aureus* in cheese samples.

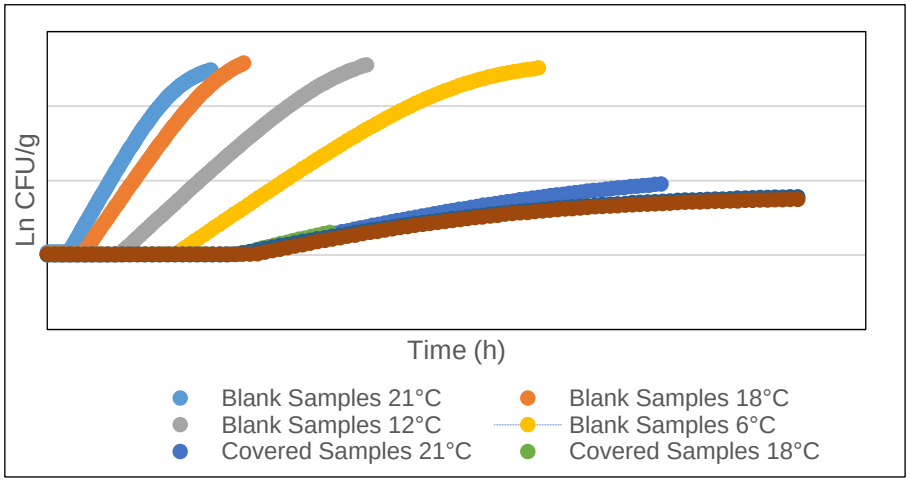

Figure 1. Growth of *S. aureus* in blank samples and cheese covered with active film

Secondary modeling

The μmax values calculated at different temperatures employing the Huang model were used to develop a secondary model through a polynomial equation. The secondary model describes the effect of active film and storage temperature on *S. aureus* behavior in cheese samples. Figure 2 show that μmax values were an ascending function of the storage temperature. However, this increase was less significant ($p<0.05$) when active film was used to cover cheese samples.

Statistical indices such as accuracy (Af) and bias (Bf) have been proposed to validate polynomial models (Baranyi *et al.*, 1999). Af represents the sum of absolute differences between observed and predicted data of one parameter determinate by the secondary model. Bf is the relative deviation among observed and predicted values; moreover, this parameter allows for determining whether the model over or under-predicts microbial growth. For example, a Bf value outside the range 0.7 to 1.5 means that the model is unsuitable (Ross, 1999). whereby, a perfect agreement between predictions and observations indicates values of AF and Bf equal to 1.0 (Choi *et al.*, 2019). Another parameter suggested to validate predictive models is RSME, which compares observed values in the experiment with those calculated by the predictive model. The closer the calculated value is to zero,

the more acceptable the model is (Baranyi *et al.,* 1996).

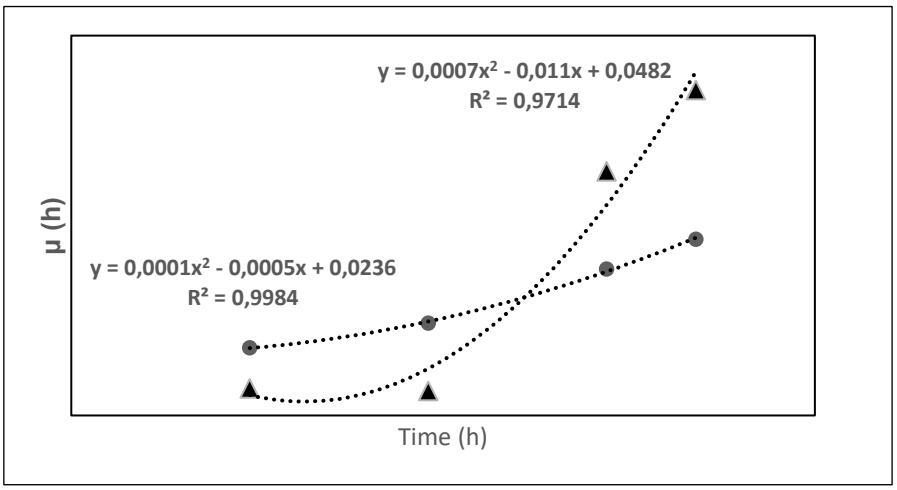

Figure 2. Effect of temperature on maximum growth rates calculated from Huang model

The mathematical validation of *S. aureus* in cheese is summarized in table 3; where, values of AF and Bf close to 1 can be seen. This indicates that both polynomial models developed herein can optimally predict *S. aureus'* growth in both blank samples and covered cheeses at different temperatures (6.0 to 21°C).

These findings are similar to those published by Yu *et al.,* (2020), who reported values of Af (1.06 – 1.13), Bf (0.91 – 1.00) and RMSE (0.00 – 0.68) in the development and validation of secondary models for *S. aureus* in raw beef under various packaging conditions. Likewise, Choi *et al*., (2019) reported values of Af from 1.06 to 1.22; Bf between 1.060 and 1.220 and RMSE from 0.160 to 1.270 in egg products model.

Regarding RSME, low values were achieved, corroborating that polynomial equations were suitable for predicting *S. aureus's* growth in a cheese sample at temperatures ranged from 6.0 to 21°C. The results from this study may be beneficial for manufacturers to formulate active films in order to apply them to dairy products, thereby reducing the risk of Staphylococcal food poisoning.

Table 3. Mathematical validation of the secondary model to describe the behavior of *S. aureus* in Colombian coastal cheese**.**

Polynomial model	Af	Bf	MSE
ln (μ) = 0,0001x2 - 0,0005x + 0,0236 Blank samples	1.094	0.913	0.006
In (μ) = 0,0007x2 - 0,011x + 0,0482 Cheese covered with active film	1.058	0.987	0.009

CONCLUSION

A secondary model was developed to predict the growth of *S. aureus* in cheese samples covered with active film and stored at both cooling and abuse temperatures. The validation process yielded accuracy and bias factors of approximately 1.0; Simultaneously, values of RSME were close to zero, indicating that polynomial models can predict the *S. aureus* growth in cheese covered with active film proving highly valuable for optimizing packaging processes that involve this kind of microorganism. The active film was manufactured employing an aqueous extract of *G. sepium*. The main compounds found in aqueous extract of *G. sepium* were: 9.12.15-octa catrienoic acid, 2.4.6-Cycloheptatrienone and Pyrocatechol, which may have bacteriostatic activity against *S. aureus*. The results of this work confirmed that aqueous extract of *G. sepium* had great potential as a natural antimicrobial. These models may be valuable for the prevention of food intoxication or cheese spoilage. Additional studies should be developed to confirm the potentiality of active gellam film with aqueous extract in order to use it as a preservative in real food systems.

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