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ORIGINAL ARTICLE

Coupling effects of structure, oxygen availability and temperature on microbial growth in a pastry filling

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Abstract

The structure of real food is a key factor to be considered in order to control microbial growth. A pastry filling has been employed as model food to study the growth of *Staphylococcus* under different conditions. Additionally, the structure of the food system has been characterised by means of rheological measurements. Frequency sweeps showed that, in all cases, the elastic component determines the rheological behaviour of model pastry filling ($G' > G''$). Values obtained for the coordination number (z) and the proportional coefficient (A) indicated that the model food exhibits more aggregate structures and stronger links at lower temperatures. According to the maximum specific growth rates, the *Staphylococcus* growth in the model pastry filling was clearly conditioned by oxygen diffusion, which is limited by the food matrix, and also by the incubation temperature. In addition, the analysis of *Staphylococcus* growth at different temperatures suggested the influence of the pastry filling structure on microorganism behaviour.

Keywords: Microorganisms; food safety; pastry; rheology; *Staphylococcus*; structure

1. Introduction

With increasing globalization, food safety has become a major concern of governments, food industry and consumers. Indeed, foodborne diseases along with waterborne diseases are nowadays the more widespread public health problem, being responsible for the death of approximately 1.8 million people every year and entailing great economic losses (Baptista *et. al*, 2017). Hence, food safety remains as industry's number one priority (Chinyama, 2014).

Among the possible origins of foodborne illness, microbiological sources stand out for entailing a great risk to public health. *Staphylococcus aureus* is an extremely versatile opportunistic pathogen that has the ability to grow and produce heat-stable toxins in food products. Meat, eggs and dairy products, as well as cream-filled bakery products are examples of food involved in staphylococcal poisoning (Alhashimi *et. al*, 2017; Silva *et al.*, 2017). *S. aureus* is often found as a commensal on skin, skin glands and mucous membranes; certainly, 30 % to 50 % of individuals are asymptomatic carriers. Therefore, the presence of these bacteria in food occurs frequently due to an inappropriate manipulation of food (Alhashimi *et. al*, 2017), including improper use of temperature during processing and conservation, cross contamination, poor personal hygiene and inadequate equipment (Rebouças *et al.*, 2017).

The majority of real food are solid or semi-solid structured systems where microorganisms are located and constrained to grow as three-dimensional colonies inside the food matrix (Aspridou *et. al*, 2014; Baka *et al.*, 2017). Due to diffusional limitations of oxygen and nutrients and

accumulation of metabolic products, these cells could experience alterations in cell development, morphology and membrane permeability, which can modify the microorganism response to various environmental factors (affecting, for instance, the thermal inactivation tolerance or the antimicrobial resistance of the bacterium) (Aspridou *et. al*, 2014; Costello *et al.*, 2018). Hence, food microstructure becomes a key factor in order to control the microbial growth in food, and thereby affects food safety in many ways.

Food model systems are an alternative for experimental data collection, where all physicochemical and structural parameters can be controlled, so results could be transferable to food products with similar properties (Aspridou *et. al*, 2014). Commonly the employed food model systems are homogeneous media with one single phase, composed by the addition of gelling agents, such as agar, in a nutrient broth, which not always mimic the characteristics of real food. As far as we know, very few studies that use more complex food models containing ingredients actually employed in risky foodstuffs have been carried out (Costello *et al.*, 2018). In this work, a first approach to determine the effect of food microstructure on the growth of *Staphylococcus* in pastry products has been carried out. With this aim, three of the ingredients most frequently used in pastry products (margarine, egg yolk and sugar) have been employed to obtain a model pastry filling. Specifically, these are basic ingredients of the filling used to prepare "brazo de Fabiola", a Spanish dessert, and, usually this filling is not cooked. The structure of the food system was characterised by means of rheological measurements and the evolution of *Staphylococcus* in the media has been monitored with the aim of identifying the effect of the structure on microorganism growth at different conditions of storage.

2. Materials and methods

2.1. Microorganism

Staphylococcus warneri (CECT 236) supplied by the Spanish Collection of Type Cultures was used as model bacterium.

2.2. Pastry filling preparation and inoculation

The pastry filling consists of three ingredients: shell eggs, sugar and margarine (sunflower oil, water, palm and coconut oil, skimmed milk powder, salt (0.3 %), aromas, potassium sorbate, citric acid, vitamins A, E and D and carotenes) (total fat content: 60 g per 100 g) which were purchased at a local supermarket and employed before the ending of their "best-before date". The model food was prepared by mixing 100 g of sugar, 250 g of margarine and the preinoculum pellet resuspended in 20 g of raw egg yolk. The preinoculum was prepared from a refrigerated stock of Petri dishes as described in Sanchez *et al.* (2019). Sterile 12 mL syringe-bodies (1.5 cm in diameter and 7.6 cm in length; HSW Norm-Ject) were packed with approximately 10 g of this inoculated medium, reaching inside 4 cm in height. Then, the syringes were closed by placing a cotton wool plug on the top and an aluminium foil sealed with Teflon® at the syringe tip (Noriega *et al.*, 2010a; Sanchez *et al.*, 2019). The initial concentration of microorganism was approximately 1×10^7 CFU g^{-1}. All the procedure was carried out in sterile conditions.

The ingredients were not sterilised, for this reason a negative control (pastry filling without being inoculated) were also included in the study (data not shown). Control experiments were carried out at the three assayed temperatures and the microbial concentrations were in these cases always below 1×10^3 CFU g^{-1} (four order lower than the initial concentration of *S. warneri*).

2.3. Experimental conditions and sampling

Syringes were incubated at different temperatures (6 °C, 20 °C and 30 °C) to simulate refrigeration and room temperatures. With the aim of evaluating the effect of oxygen availability, sampling was carried out by taking 1 g of the model pastry filling at two longitudinal positions of the syringe: 3.7 cm to 4.0 cm (surface) and 0.0 cm to 0.3 cm (bottom). All samples were taken in triplicate (from three different syringes). Each sample was transferred to stomacher bags containing 9 mL of sterile saline solution (0.7 % NaCl) and serial decimal dilutions of the saline solution were plated in triplicate onto Nutrient Broth Agar. Petri dishes were incubated at 30 °C for 48 h before counting. All the procedures were carried out in sterile conditions.

2.4. Characterisation of the structured media

A HAAKE MARS II rotational rheometer (ThermoFisher Scientific) has been employed to carry out the rheological measurements using a plate/plate measuring system (PP60Ti) with a gap of 1 mm. To allow the stresses induced during sample loading to relax, samples rested for at least 15 min before any measurement (Laca *et al.*, 2010a). The frequency sweeps were carried out from 0.1 Hz to 10 Hz at a constant shear stress of 5 Pa (within the linear viscoelasticity range). Model foods were measured at different temperatures (6 °C, 15 °C, 20 °C, 25 °C and 30 °C). Analyses were conducted at least in triplicate.

3. Estimation of maximum specific growth rate

Maximum specific growth rate was obtained according the next equation:

$$\frac{dx}{dt} = -\mu_{\max} x \tag{1}$$

Therefore, the following linear function was fitted to the microbial growth data obtained from plate counting:

$$\ln x = \ln x_0 - \mu_{\max} t \tag{2}$$

Where x_0 and x are the microbial concentration in the medium at initial time and at t time, respectively. Since this linear relation is exclusively valid during the exponential growth phase (when cells are dividing regularly by binary fission and the growth rate is proportional to microorganism concentration), only the data corresponding to the first part of the curve were employed for the fitting. The data were selected by means of the fitting goodness, i.e., those data that gave values of r^2 below 0.95 were assumed to be out of the exponential growth phase and were not considered.

3.1. Statistical analysis

Excel software was employed to carry out a one-way ANOVA with a 95 % confidence interval to analyse the data.

4. Results and discussion

4.1. Effect of temperature

The model pastry filling prepared with eggs, margarine and sugar, as explained in Material and Methods section, resulted in an accurate medium for *S. warneri* growth at all the assayed temperatures (**Figure 1**). In all cases, the initial inoculum was in the order of magnitude of

1×10^7 CFU g^{-1}. In the six days that the experiment lasted, the concentration of bacteria increased around two orders of magnitude in the surface of the syringes when they were incubated at 30 °C, achieving values higher than 1×10^9 CFU g^{-1}. The bacterial growth was slightly lower at 20 °C and at 6 °C microorganism concentration only increased in one order of magnitude. In this case, the maximum value, that was not over 1×10^8 CFU g^{-1}, was achieved after 80 h of incubation. These results are not in accordance with those found by Ananou *et. al* (2018) who reported that when *S. aureus* was incubated in liquid yolk at refrigerated temperatures the concentration of viable bacteria remained constant during 96 h. Similarly, and also in liquid yolk, Sanchez *et al.* (2019) found that the concentration of *S. warneri* was approximately the same during 72 h at 11 °C. This can be explained mainly by the different composition of the media, since the model pastry filling contained a high concentration of sugar that favoured microorganism growth. The effect of temperature on *Staphylococcus* growth are easily reflected by comparing the maximum specific growth rates (μ_{max}) (**Table 1**). The values obtained for the surface of the food model at 6 °C, 20 °C and 30 °C were 0.0209 h^{-1}, 0.0438 h^{-1} and 0.0573 h^{-1}, respectively. According to ANOVA results, there were statistically significant differences between the μ_{max} values obtained

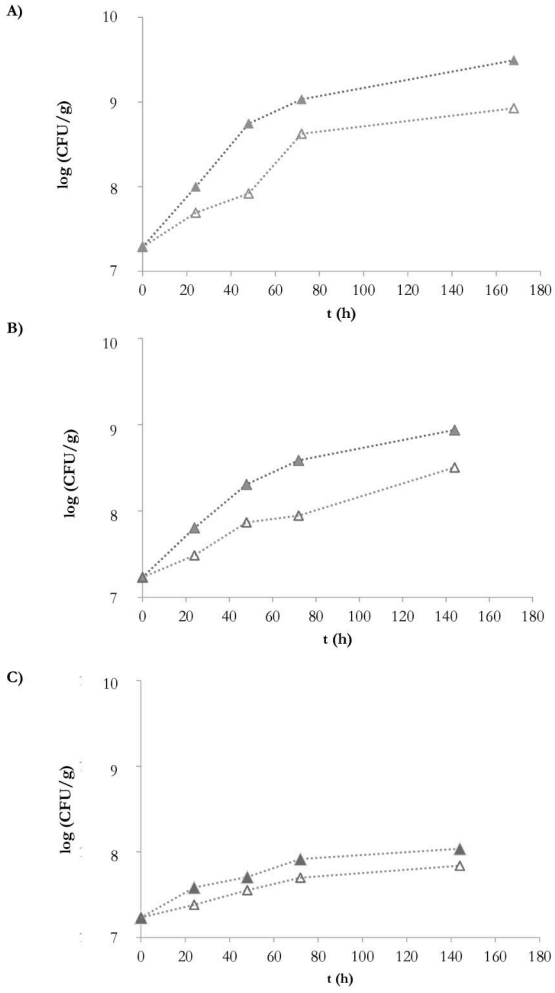

Figure 1. Growth of *S. warneri* in model pastry filling at different temperatures: A) 6 °C, B) 20 °C and C) 30 °C. Surface (▲) and bottom (△) samples. In all cases, relative standard deviation < 5 %.

for the different temperatures of incubation. Additionally, the maximum specific growth rates obtained at surface and bottom for each temperature studied were also significantly different (95 % confidence interval).

In addition, it should be pointed out that at all the analysed temperatures lag phase was not observed, although it might take place at the very first hours of incubation. Specifically, at room temperatures in only 24 h the bacterial concentration increased almost in one order of magnitude. It is evident the importance of storing this kind of pastry products at refrigerated conditions just after being prepared. However, it should be taken in mind that microbial growth is not inhibited even at refrigerated temperatures.

5. Effect of oxygen availability

Although *S. warneri* can survive in presence or absence of oxygen, the environmental oxygen level determines the metabolism of this facultative bacterium (Wallace *et al.*, 2016; Sanchez *et al.*, 2019). The presence of diffusional limitations plays an important role in solid systems, so oxygen is the main factor to limit microbial growth in solid food. Different works have studied the mechanisms and conditions that determine the behaviour of facultative bacteria, mainly *Listeria*, in food environments with low oxygen concentration and solid systems. Specifically, Lungu *et al.* (2009) carried out a review on the effect of low oxygen and/or anaerobiosis in the growth, survival and proliferation of this pathogen in food. *Listeria* behaviour has been studied in different foodstuffs, such as cheese, chicken and fish and, in all cases, structure was a key parameter regarding this genus growth (Baka *et al.*, 2017; Noriega *et al.*, 2008, 2010b; Verheyen *et al.*, 2018). Additionally, Aspridou *et. al* (2014) evaluated the growth of *L. monocytogenes* in gelling media and reported that pathogen's growth was lower in microstructured media, resulting in some cases in microbial growth suppression. The effect of oxygen availability and diffusional limitations on the genus *Staphylococcus* growing in food has been much less studied (Alonso *et. al*, 2021; Belay and Rasooly, 2002; Sanchez *et al.*, 2019).

With the aim of knowing the effect of the availability of oxygen on the growth of *S. warneri* in the model pastry filling, in addition to the samples taken on the surface, samples of the bottom of the syringes were also obtained. As can be seen in Figure 1, the growth of *S. warneri* in all analysed temperatures was higher in the surface position than in the bottom position. After 48 hours of incubation the amount of CFU/g in the surface was almost one order of magnitude

Table 1. Maximum specific growth rate (μ_{max}) values at the exponential phase of growth of *S. warneri* in model pastry filling at different temperatures and positions. In all cases $r^2 > 0.95$.

Temperature	Longitudinal position	μ_{max} (h^{-1})
30 °C	Surface	0.0573^a
	Bottom	0.0407^b
20 °C	Surface	0.0438^c
	Bottom	0.0242^d
6 °C	Surface	0.0209^e
	Bottom	0.0150^f
Different letters indicate significant differences between groups ($p < 0.05$)		

higher than values measured in the bottom in samples stored at 30 °C. However, in model pastry filling stored at 6 °C the difference between surface and bottom was much lower. The lower μ_{max} of *Staphylococcus* at this temperature implied a lower consumption rate of oxygen, so that the effect of diffusional limitations was less important for lower temperatures; probably, because temperature at this condition is a more limiting factor than oxygen availability. In Table 1, it can be observed that μ_{max} values were in all cases higher for surface positions than for bottom positions. Diffusional limitations imply lower concentration of oxygen in the inner part of the food, which reduce the maximum specific growth rate of *S. warneri*. Nevertheless, the growth is not inhibited at any of the studied temperatures.

5.1. Structural characterisation of the model food. Does the structure affect the microorganism growth?

Rheological assays have been carried out in order to characterise the model pastry filling at different temperatures. In **Figure 2** it is shown the values of storage (G') and loss moduli (G'') obtained from frequency sweeps at different temperatures, and in **Table 2** it is shown the average values for G' and G'' and their corresponding average $\tan\delta$ values. ANOVA showed that there were statistically significant differences between the means of G' and G'', and the moduli values increased as temperature decreased, which indicates that the filling structure becomes stronger with lower temperatures. In all cases, since the storage modulus is much larger than the loss modulus, being $\tan\delta < 1$, the elastic component dominates the rheological behaviour of the model pastry filling. The storage modulus increased slightly with increasing frequency

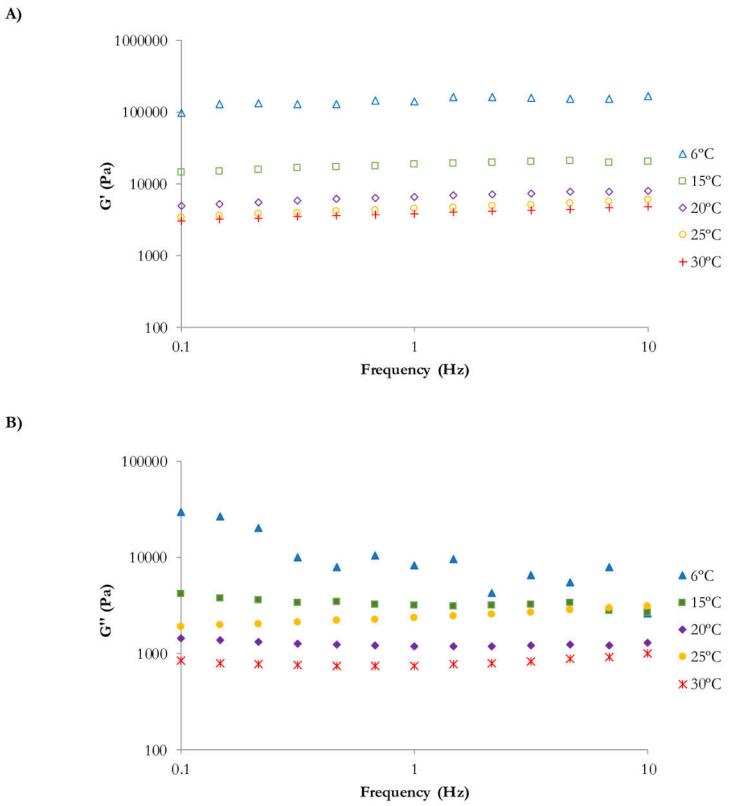

Figure 2. Rheological characterization of the model pastry filling: A) Storage modulus (G') and B) Loss modulus G'' as a function of the angular frequency at 6 °C, 15 °C, 20 °C, 25 °C and 30 °C.

Table 2. Rheological characterization of the model pastry filling obtained from frequency sweeps. Average values for storage modulus (G'), loss modulus (G'') and their associated average standard deviations, and average tan δ values.

Temperature	G' (kPa)	G'' (kPa)	tan δ
6 °C	143.8 ± 19.3^a	11.5 ± 8.5^a	0.0801^a
15 °C	18.2 ± 2.2^b	3.3 ± 0.4^b	0.1824^b
20 °C	6.6 ± 1.0^c	1.3 ± 0.1^c	0.1918^c
25 °C	4.6 ± 0.8^d	2.4 ± 0.4^d	0.5317^d
30 °C	3.9 ± 0.5^e	0.8 ± 0.1^e	0.2113^e

Different letters within columns indicate significant differences between groups ($p < 0.05$)

for all the temperatures considered, which is a common behaviour of semi-solid fats and it is in accordance with results reported by other authors for different vegetal butters (Emadzadeh *et al.*, 2013; Gregersen *et al.*, 2015; Muresan *et al.*, 2014). The values of the loss modulus remained also constant over the frequency range, except for the analysis carried out at 6 °C. At this temperature, the value of G'' slightly decreased from 0.1 Hz to 0.5 Hz and from 0.5 Hz to 10 Hz values fluctuated between approximately 8000 Pa and 10 000 Pa. In general, the standard deviations associated with the average values of moduli increased with decreasing temperature, which reflected a less homogeneous structure at lower temperatures.

The differences observed in G' and G'' indicate that the food model structure notably varies depending on temperature. In addition, the frequency dependency may be originated by the result of interchain entanglements not having enough time to break or by the result of the small deformation mechanical working, causing structural changes in the fat samples (Gregersen *et al.*, 2015). In order to obtain a more detailed information about structural modifications, frequency sweep tests data were fitted to equation 1, which is commonly used to characterise emulsions and gel foods (Gabriele *et al.*, 2001; Laca *et al.*, 2010b; Matos *et al.*, 2018):

$$G^* = A \cdot v^{1/z} \tag{3}$$

where G^* is the complex modulus in Pa, the frequency in Hz, z (dimensionless) the coordination number and A (G^* in Pa at 1 Hz) the proportional coefficient. The coordination number (z) is a measure of the number of rheological units correlated with one another in the three-dimensional structure, whereas the proportional coefficient (A) is related to the strength of the interaction between those units (Mancini *et al.*, 2002).

Values of parameters A and z are shown in **Table 3**. The proportional coefficient value varied from 3.9 kPa to 96.8 kPa $s^{1/z}$, whereas the variation range of the coordination number was much narrower (10.2 to 14.3). In addition, and according to ANOVA results, there were statistically significant differences between the A values, on the contrary, the z values were not significantly different (95 % confidence interval).

The value found for A at 25 °C was 4.7 kPa $s^{1/z}$, much lower than that reported by Muresan *et al.* (2014) for different particle size of sunflower tahini at this temperature (104 kPa $s^{1/z}$ to 443 kPa $s^{1/z}$). On the contrary, the z value was quite higher than that reported by the former authors (2.4 to 3.5). With respect to the network strength, a clear trend can be observed, since A value raised when temperature decreased. Similar network extensions (z) were found for 30 °C, 25 °C

Table 3. Power-law parameters obtained from frequency sweeps. Average values ± SD are reported. In all cases, except for 6 °C, $r^2 \geq 0.91$.

Temperature	Power-law parameters	
	A (kPa $s^{1/z}$)	z
6 °C	96.8 ± 65.3^a	14.3 ± 3.8^a
15 °C	18.4 ± 4.5^b	13.6 ± 1.6^a
20 °C	6.6 ± 1.3^c	10.2 ± 0.5^a
25 °C	4.7 ± 0.4^d	10.8 ± 2.4^a
30 °C	3.9 ± 2.4^e	10.5 ± 1.0^a
Different letters within columns indicate significant differences between groups ($p < 0.05$)		

and 20 °C, whereas the z value slightly increased at 15 °C and 6 °C. Thus, in general, samples at lower temperatures show more aggregate structures and stronger links compared to those at warmer ones. These results are in agreement with those reported by Migliori *et al.* (2009) who analysed the effect of egg as ingredient in Yorkshire pudding batter. They found that parameter z values were similar at 10 °C and 30 °C (2.4 and 2.6, respectively), whereas A decreased from 21 to 12 at 10 °C and 30 °C, respectively.

Rheological data indicated that the structure of the model pastry filling was very similar at 20 °C and 30 °C, whereas it notably changed at 6 °C (see Figure 2 and Table 3). In order to go in depth about the possible effect of the structure over the growth rate of the bacterium, the maximum specific growth rates obtained at the different temperatures for the surface and bottom positions (Table 1) were fitted to an Arrhenius type equation (Peleg *et al.*, 2012). When $\ln \mu_{\max}$ was depicted versus $1/T$, the linear fitting was not very good for any of the two positions, with R-squared lower than 0.98 **Figure 3**). This suggests that the microorganism growth not only was affected by temperature and oxygen availability, but also by the confinement of the bacteria inside the structured media, which was formed by a network much stronger at 6 °C. In agreement with these results, Aspridou *et. al* (2014) reported that the microstructure of the medium could cause a decrease in the growth rate of *Listeria monocytogenes*. A complicated relation among temperature, composition and structural aspects of food model systems exists, as it was also indicated by Baka *et al.* (2017) who studied the behaviour of *L. monocytogenes* on fish-protein based model systems.

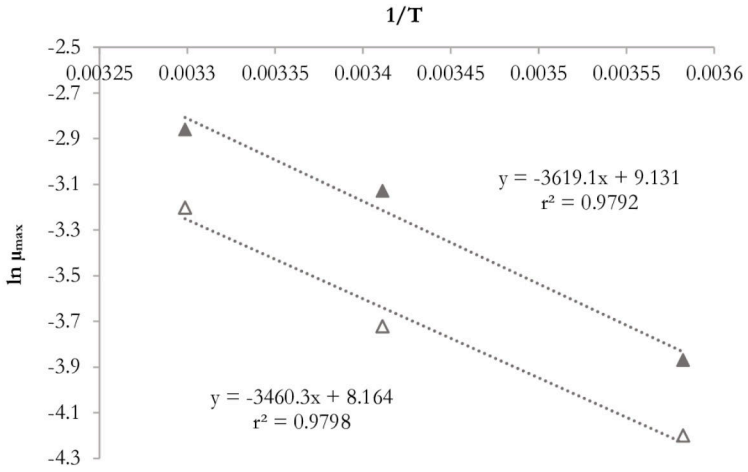

Figure 3. Fitting of the maximum specific growth rates of *S. warneri* (surface: ▲ and bottom: △) at different temperatures to an Arrhenius type equation: $\mu_{\max}$ in h^{-1} and T in K.

6. Conclusions

The growth of *Staphylococcus* in solid media was conditioned by oxygen diffusion and also by temperature, which was clearly reflected by the values obtained for the maximum specific growth rates at the exponential phase.

Frequency sweep results showed that the elastic component dominates the rheological behaviour of model pastry filling. In addition, A and z parameters indicated that the network strength increased when temperature decreased, whereas network extensions slightly increased at lower temperatures.

The values obtained for the $\mu_{\max}$ at different temperatures were fitted to an Arrhenius type equation. The moderate goodness of this fitting suggests that the structure of the model pastry filling exerted certain influence on the behaviour of *Staphylococcus*.

The results of this work revealed a complex relationship between system structure and other factors such as temperature and food model composition. A deep knowledge of this relationship would favour to obtain reliable predictions of the growth kinetics of bacteria in real food products, which could help to avoid the proliferation of undesirable or even pathogenic microorganisms in different foodstuffs.

7. Acknowledgements

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8. Conflict of interest

The authors certify that they have no conflict of interest.

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Efectos acoplados de la estructura, disponibilidad de oxígeno y temperatura en el crecimiento microbiano del relleno de pastelería

Resumen: La estructura de los alimentos es un factor clave a considerar con miras a controlar el crecimiento microbiano. Se empleó un relleno de pastelería como alimento modelo para estudiar el crecimiento de Staphylococcus bajo diferentes condiciones. Además, la estructura del sistema se caracterizó con base en mediciones reológicas. Los barridos de frecuencia mostraron que, en todos los casos, el componente elástico determina el comportamiento reológico del relleno de pastelería usado como modelo ($G' > G''$). Los valores obtenidos para el número de coordinación (z) y el coeficiente proporcional (A) indicaron que el alimento modelo exhibe más estructuras de agregados y enlaces más fuertes a temperaturas más bajas. De acuerdo con las máximas tasas de crecimiento específico, el crecimiento de Staphylococcus en el modelo de pastelería estuvo claramente condicionado por la difusión de oxígeno, que está limitada por la matriz del alimento y también por la temperatura de incubación. Además, el análisis del crecimiento de Staphylococcus a distintas temperaturas sugirió la influencia de la estructura del relleno de pastelería en el comportamiento del microrganismo.

Palabras Clave: microorganismos; inocuidad de los alimentos; pastelería; reología; *Staphylococcus*; estructura

Efeitos de acoplamento entre a estrutura, a disponibilidade de oxigénio e a temperatura no crescimento microbiano em recheio de pastelaria

Resumo: A estrutura dos alimentos é um fator chave a se considerar para controlar o crescimento microbiano. Neste estudo, utilizamos recheio de pastelaria como alimento modelo para estudar o crescimento de Staphylococcus em diferentes condições. Adicionalmente, caracterizamos a estrutura do sistema alimentar mediante medições reológicas. As varreduras de frequência mostraram que, em todos os casos, o componente elástico determinou o comportamento reológico do recheio de pastelaria utilizado como modelo ($G' > G''$). Os valores obtidos para o número de coordenação (z) e o coeficiente proporcional (A) indicaram que o alimento modelo apresenta mais estruturas de agregados e uniões mais fortes a temperaturas mais baixas. Segundo as taxas de crescimento específicas máximas, o crescimento de Staphylococcus no receio de pastelaria modelo esteve claramente condicionado pela difusão do oxigénio, que é limitada pela matriz do alimento e pela temperatura de incubação. Adicionalmente, a análise de crescimento de Staphylococcus a diferentes temperaturas sugeriu que a estrutura do recheio de pastelaria influencia o comportamento dos microrganismos.

Palavras-chave: microorganismos; segurança alimentar; pastelaria; reologia; *Staphylococcus*; estrutura.

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