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Mechanisms of interaction between *Candida albicans* and *Streptococcus mutans*: An experimental and mathematical modelling study

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Abstract

Objective. To evaluate the mechanisms of microbial interaction between the oral pathogens *Candida albicans* and *Streptococcus mutans.* ***Materials and methods.*** Growth kinetics for the two micro-organisms, cultured individually or together, were followed experimentally for 36 h. The different growth curves were analysed by means of mathematical modelling. ***Results.*** Under the experimental conditions, *S. mutans* final concentration, when grown individually, was 5-times that of *C. albicans.* Contrarily, when both micro-organisms grew together, this ratio was inversed and *C. albicans* final concentration was even higher than that of *S. mutans.* When both micro-organisms share the niche, a model including linear competition among one another was best suited to reproduce the experimental observations. The results of this model show that the initial growth rates of both species are positively influenced by their mutual interaction. However, at longer incubation times, *C. albicans* prevents bacterial growth and achieves concentrations 4-times higher than when grown individually. ***Conclusions.*** The results suggest that *C. albicans* biofilm formation could be potentiated by the presence of *S. mutans* by two mechanisms: synergically at short times and by competition at longer periods.

Key Words: *oral microbiology, biofilms, Streptococcus, Candida, computational models*

Introduction

The oral cavity represents a host environment with specific characteristics that favour the development of a great variety of micro-organisms. In this sense, the mult-species nature of dental plaque makes the oral microbial community one of the best biofilm models for studying inter-species interactions [1]. Furthermore, our understanding of the mechanisms and factors that regulate such interactions could provide valuable information for the proper management of periodontal diseases, e.g. dental caries and periodontitis [2,3].

In this work, the interaction between two usual inhabitants of the buccal microbiota was investigated: the bacteria *Streptococcus mutans* and the yeast *Candida albicans*. *S. mutans* is considered as one of the main aetiological agents of human dental caries [4,5], while candidiasis represents the most common oral fungal infection in humans [6]. Streptococci can localize into the dorsal surface of the tongue, teeth and other mucosal surfaces or remain free in saliva [7], while *Candida* species are frequently indentified on the tongue, palate and jugal mucosa [8]. Several studies have demonstrated the growing capability of *C. albicans* and *S. mutans* separately, but only a few reports have addressed the problem of their survival when both species simultaneously colonize the same niche [9–11]. Although the development of caries is initially related to the formation of the cariogenic film by *S. mutans*, the progression of established lesions can include other micro-organisms such as

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C. albicans [12]. Additionally, a recent study suggests that the occurrence of dental cavities in children is positively correlated with the frequency of oral candidal carriage [13].

To further study the potential interaction between *C. albicans* and *S. mutans*, two approaches are presented here. First, the experimental growth kinetics of these two micro-organisms were investigated during 36 h, when they were grown individually or together. Second, we propose the use of mathematical modelling to describe the mechanisms of interaction between both micro-organisms.

Materials and methods

Micro-organisms and growth conditions

Individual cultures of *C. albicans* (ATCC 10231) and *S. mutans* (ATCC 35668) or cultures of both micro-organisms growing together were studied. To isolate individual colonies, *C. albicans* yeast were seeded in Sabouraud Dextrose Agar (Sigma-Aldrich, Germany) and incubated under aerobic conditions at 37°C for 18 h. Similarly, *S. mutans* bacteria were seeded onto Todd-Hewitt Agar (Difco, BD, Franklin Lakes, NJ, USA) under micro-anaerobic conditions at 37°C for 24 h. Afterwards, individual colonies of each micro-organism were resuspended in liquid media composed of 0.25 ml of Sabouraud Broth, 0.25 ml of Todd Hewitt Broth and 0.5 ml of Basal medium during 5 h. Basal medium composition: Tripticase, 20 g; NaCl, 2 g; $MnSO_4$, 15 mg; $MgSO_4$, 10 mg; K_2POH, 3 g; $KPOH_2$, 2 g, K_2CO_3, 1 g, H_2O_d, 1000 ml, pH = 7. For the experimental growth curves and following the rationality described in Brusca et al. [9], samples of each micro-organism from exponential phase growing cultures were taken, quantified spectrophotometrically and adjusted by dilution into 1 ml of fresh liquid media (0.25 ml of Sabouraud Broth, 0.25 ml of Todd Hewitt Broth and 0.5 ml of Basal medium). Growth curves were carried out at 37°C without agitation and using small neck flat-bottomed vials with a diameter and height of 20 mm and 45 mm, respectively. No changes in pH (pH = 7) were observed during the measurement period. *C. albicans* and *S. mutans* colonies have different phenotype and were quantified by microscopic observation of colonies adherence to the bottom of the vials. Additionally, Gram staining and Rapid ID 32 Strep and API 20 C. AUX (BioMérieux, France) identification kits were used to confirm the identity of the colony-forming units (CFU) followed during the growth curves. Growth was monitored at regular intervals during 24 h and a final measurement at 36 h was performed to confirm that steady state conditions were reached. All growth curves were done in triplicate and data points are represented as mean ± standard deviation.

Mathematical models, simulations and fitting procedure

In order to describe the growth behaviour of the two studied micro-organisms, three types of models are considered; namely logistic, co-existence and competition. The time evolution of CFU.ml^{-1}, $N(t)$, for each individually growing micro-organism was studied considering a logistic-growth mathematical model (logistic model):

$$\frac{dN_i}{dt} = r_i . N_i . \left(1 - \frac{N_i}{K_i}\right) \qquad (1)$$

where N_i represents the number of CFU per millilitre of the i species (*C. albicans* or *S. mutans*) while dN_i/dt corresponds to the growth rate for the i species. The parameters r_i and K_i represent the growth rate constant and the carrying capacity, respectively, of the i species. Solving equation (1) we obtain:

$$N_i(t) = \frac{K_i}{\left(1 + \left(\frac{K_i - N_i^0}{N_i^0}\right) e^{-r_i . t}\right)} \qquad (2)$$

where the constant N_i^0 represents the number of CFU.ml^{-1} of the i species at initial time.

The coexistence model was defined as:

$$N_{c/s}(t) = \frac{K_{c/s}}{\left(1 + \left(\frac{K_{c/s} - N_{c/s}^0}{N_{c/s}^0}\right) e^{-r_{c/s} . t}\right)} \qquad (3)$$

$$N_{s/c}(t) = \frac{K_{s/c}}{\left(1 + \left(\frac{K_{s/c} - N_{s/c}^0}{N_{s/c}^0}\right) e^{-r_{s/c} . t}\right)} \qquad (4)$$

where subscripts *c/s* and *s/c* refer to *C. albicans* growing in the presence of *S. mutans* and *S. mutans* growing in the presence of *C. albicans*, respectively.

Finally, the competition model was defined as:

$$\frac{dN_{c/s}}{dt} = r_c N_{c/s} \left(1 - \frac{N_{c/s} + \alpha . N_{s/c}}{K_{c/s}}\right) \qquad (5)$$

$$\frac{dN_{s/c}}{dt} = r_s N_{s/c} \left(1 - \frac{N_{s/c} + \beta . N_{c/s}}{K_{s/c}}\right) \qquad (6)$$

where the linear terms $\alpha.Ns/c$ and $\beta.Nc/s$ represent the competition between *S. mutans* and *C. albicans*.

α represents the competition effect over *C. albicans* due to *S. mutans*, while β represents the effect exerted over *S. mutans* by *C. albicans*.

Each model was simulated and then fitted to the experimental data. Algebraically, the fitting procedure consists in the exploration of the free parameters space in order to minimize a given function; in this case, the sum of the squares of the residues between the experimental data, CFU.ml^{-1} as function of time ($N(t)$) and that simulated by the model under study.

For the logistic model, model-dependent fit was carried on using a program developed in Fortran 95 code (code and binaries available by request), which calculates analytically the growth kinetics for each micro-organism *i*. The algorithm performs simulations of $N_i(t)$, searching the values of the parameters (N^0_i, K_i and r_i) that minimize the sum of the square distance between simulated and experimental data ($S_{\min}$).

The co-existence and the competition models were fitted simultaneously to three sets of experimental data (each species growth in the presence of the other and the sum of the total number of colonies). The co-existence model was fitted to the experimental data using the Fortran 95 algorithm previously described. For the competition model the time course of $N_i(t)$ (in CFU.ml^{-1}) for each micro-organism *i* was simulated, solving its system of equations using the fourth order Runge-Kutta method. The sum of squares of the distances between modelled and experimental values of the sum of $N(t)$ for the species under study was calculated and minimized ($S_{\min}$), varying the parameter values using the Solver package of Microsoft Excel.

The quality of the fitting for the different models assayed was analysed in terms of the distribution of regression residuals, the Akaike information criterion (*AIC*) and the delta *AIC* (ΔAIC) criterion [14–16]. The regression residuals are defined as the difference between the experimental values and those predicted by the simulated curves and used to evaluate, by visual inspection, how well the model described the experimental data. The *AIC* index permits to select the model that best fits the experimental data with the minimum number of parameters. The *AIC* index is defined as:

$$AIC = n \cdot \ln\left(\frac{S_{\min}}{n}\right) + 2np \qquad (7)$$

where n and p are the number of experimental data points and the free parameters of the model, respectively. The model showing the lowest value of *AIC* can be initially assumed as the best model to describe the experimental data. Finally, the ΔAIC criterion allows to compare the best model to the rest of the tested models and estimating the probability of other models to be also considered as good candidates [16]. The ΔAIC can be defined as:

$$\Delta AIC = AIC_{Coex} - AIC_{Comop} \qquad (8)$$

where AIC_{Coex} and AIC_{Comp} are the *AIC* values for the co-existence and the competition models, respectively. If ΔAIC takes values below 2, both models have similar probability of being correct.

Steady state solution for each mathematical model

The stationary solutions for the co-existence and competition models developed through this work can be simply calculated constituting non-trivial equilibrium points ($N_{s/c}{}^{\star}$, $N_{c/s}{}^{\star}$, where the superscript '$\star$' represents the steady state conditions). For the co-existence model it is immediately obvious that $K_{s/c}$ and $K_{c/s}$ will represent the equilibrium points. On the other hand, according to equations (5) and (6), the steady state solutions for the competition model are given by the following expressions:

$$N^*_{c/s} = \left(\frac{K_{c/s} - a.K_{s/c}}{1 - a\beta}\right) \qquad (9)$$

$$N^*_{s/c} = \left(\frac{K_{s/c} - \beta.K_{c/s}}{1 - a\beta}\right) \qquad (10)$$

Results

Growing kinetics of C. albicans and S. mutans: Experimental data

The time course of colony-forming units (CFU) per millilitre, $N(t)$, was measured to quantify the growth of *C. albicans* and *S. mutans*, incubated separately or together (Figures 1 and 2). When growing individually, the growth rate during the exponential phase was faster for *S. mutans* than for *C. albicans*. Additionally, *S. mutans* reached a higher stationary level of $N(t)$ compared to *C. albicans* (Figure 1).

Both species experienced a change in their growth kinetics when co-cultured. *C. albicans* increased its growth rate during exponential phase and attained a higher concentration during the stationary phase in the presence of *S. mutans* (Figure 2A). On the other hand, *S. mutans* showed also a higher growth rate during the exponential phase, but a lower stationary level when *C. albicans* was present (Figure 2B). Additionally, the $N(t)$ of *S. mutans* in the presence of *C. albicans* showed a non-monotonic behaviour; reaching a temporal maximum at ~ 12 h of incubation and decreasing afterwards to a stationary value.

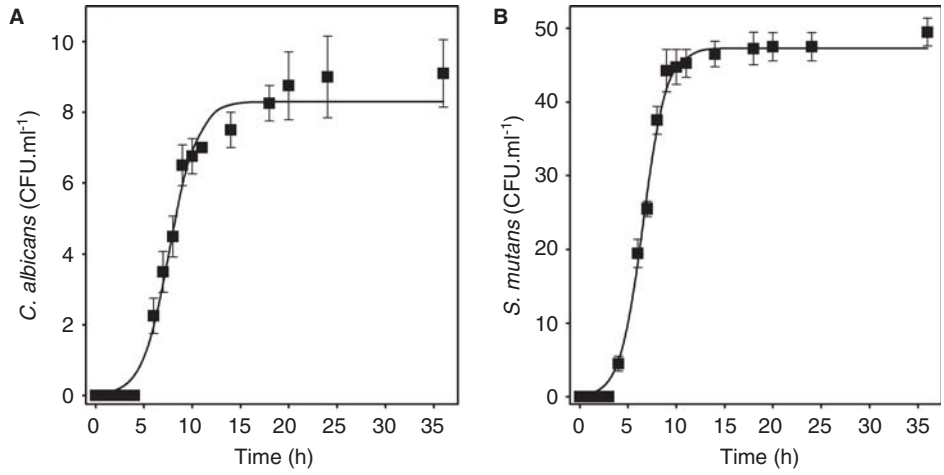

Figure 1. Mathematical modelling of *C. albicans* and *S. mutans* growth alone: the logistic model. (A) *C. albicans* vs time when growth alone. (B) *S. mutans* vs time when growth alone. Continuous curves represent model-dependent fit of the logistic model to the experimental data with the best fitting parameters depicted in Table I.

Mathematical modelling of C. albicans and S. mutans growing individually: The logistic model

To gain quantitative information from the experimental observations, a mathematical modelling approach was used. The time evolution of CFU.ml^{-1}, $N(t)$, for each species growth separately was studied considering a logistic-growing mathematical model [17]. The logistic model is widely employed in biology, ecology and other related disciplines to study population dynamics and previous work indicate that can describe satisfactorily bacterial growth in controlled conditions [18]. The solid line of Figures 1A and B shows the best simulated curves fitted to the experimental growing curves of *C. albicans* and *S. mutans*, respectively, with the best fitting parameter values depicted in Table I. Although the logistic model fails to describe the initial jump of the growth curves (≈ 4 h), the overall uniform and close distribution of the data points around the simulated curves strongly suggests that the logistic model is a good candidate to describe the growth behaviour of the two micro-organisms.

Mathematical modelling of C. albicans and S. mutans growing together: The co-existence model

Our experimental results show that both species behaved differently when they grew together compared to when they grew alone (Figure 2). Initially, we tested if we could describe the behaviour of each micro-organism by two individual logistic models combined (co-existence model). The number of colonies at zero time was fixed at the values obtained from the fittings of each organism growth individually and a global model-dependent fit, varying the parameters $r_{c/s}$, $r_{s/c}$, $K_{c/s}$ and $K_{s/c}$, was performed.

Figure 2 shows the best fitting curves obtained by simulation of the co-existence model with the best

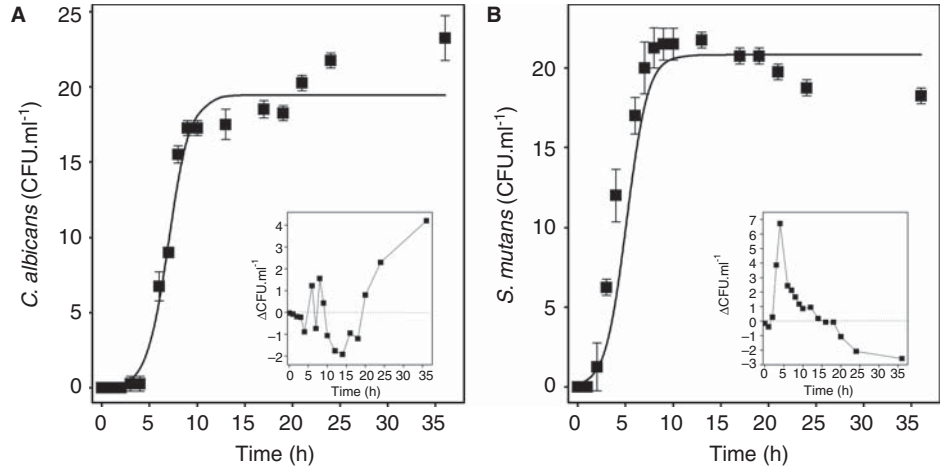

Figure 2. Mathematical modelling of *C. albicans* and *S. mutans* growth together: the co-existence model. (A) *C. albicans* vs time growth in the presence of *S. mutans*. (B) *S. mutans* vs time growth in the presence of *C. albicans*. Continuous curves represent model-dependent fit of the co-existence model to the experimental data with the best fitting parameters depicted in Table I. Insets show the regression residuals between the experimental growth curves of each micro-organism and model-dependent fitting curves.

Table I. Best fitting parameters values for the different models. Best fitting parameter values corresponding to the models describing the growing kinetics of *C. albicans* (subscript *c*) and *S. mutans* (subscript *s*) cultured individually or together.

	Growing alone	Growing together	
Parameters	Logistic model	Co-existence model	Competition model
N_c^0 or $N_{c/s}^0$ [CFU ml^{-1}]	0.03 ± 0.01	0.030	0.030
N_s^0 or $N_{s/c}^0$ [CFU ml^{-1}]	0.15 ± 0.04	0.150	0.150
r_c or $r_{c/s}$ [h^{-1}]	17.5 ± 0.4	22 ± 1	26 ± 1
r_s or $r_{s/c}$ [h^{-1}]	21.0 ± 0.2	23 ± 1	23 ± 1
K_c or $K_{c/s}$ [CFU ml^{-1}]	8.3 ± 0.2	19.4 ± 0.3	34 ± 3
K_s or $K_{s/c}$ [CFU ml^{-1}]	47.3 ± 0.3	21 ± 1	33 ± 2
α	—	—	0.68 ± 0.09
β	—	—	0.7 ± 0.2
AIC	—	38.6	39.1

fitting parameter values shown in Table I. The visual inspection of the fitting results indicates that the co-existence model was capable of describing the behaviour of both micro-organisms when growth together with a small bias over the distribution of points for *S. mutans*. This result suggests that, in a first approximation, mutual changes of the initial growth rate and carrying capacity of the media could explain the observed results. However, the co-existence model does take into account any explicit mechanism of interaction.

Mathematical modelling of the interaction mechanism between C. albicans and S. mutans: The competition model

A possible mechanism of interaction is a competition between species where they can limit the growth rate of each other [19]. To test this, we used the Lotka-Volterra model [20] that adds a linear term representing the competition between each micro-organism.

Fixing $N_{c/s}{}^0$, $N_{s/c}{}^0$ $r_{c/s}$, $r_{s/c}$, $K_{c/s}$ and $K_{s/c}$ with the best fitting values of each micro-organism grown individually and then fitting the model by varying only α and β did not allow one to obtaing a reasonable fitting (data not shown). This indicates that the model loaded with parameter values obtained from the fittings of the micro-organism growing alone but adding a competition factor could not be an explanation for the experimental data when both micro-organisms grow together. When leaving the six parameters free, $r_{c/s}$, $r_{s/c}$, $K_{c/s}$, $K_{s/c}$, α and β, the model-dependent fitting process arrived at a very good solution for $N(t)$ of each micro-organism (Figures 3A and B), with the best fitting parameter values for the competition model shown in Table I. The visual inspection of the fitting results shows a more homogenous distribution of the data points around the simulated curves when compared to the results obtained with the co-existence model.

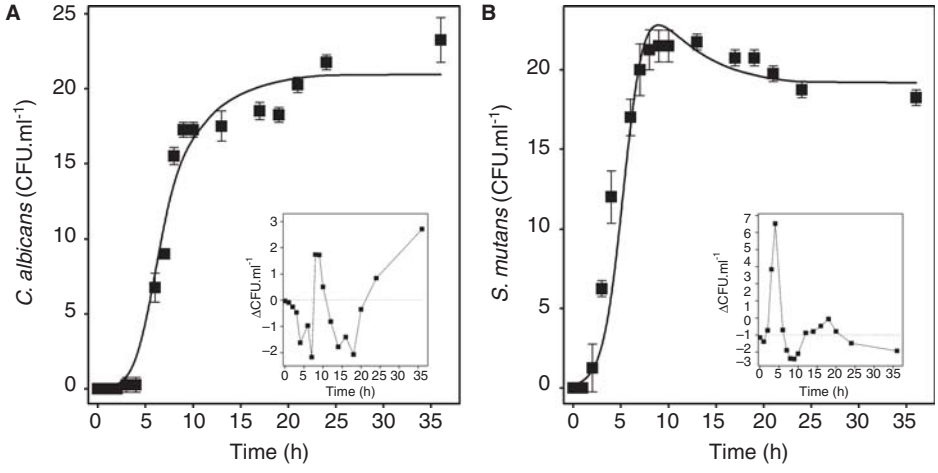

Figure 3. Mathematical modelling of *C. albicans* and *S. mutans* growth together: the competition model. (A) *C. albicans* vs time growth in the presence of *S. mutans*. (B) *S. mutans* vs time growth in the presence of *C. albicans*. Continuous curves represent model-dependent fit of the competition model to the experimental data with the best fitting parameters depicted in Table I. Insets show the regression residuals between the experimental growth curves of each micro-organism and model-dependent fitting curves.

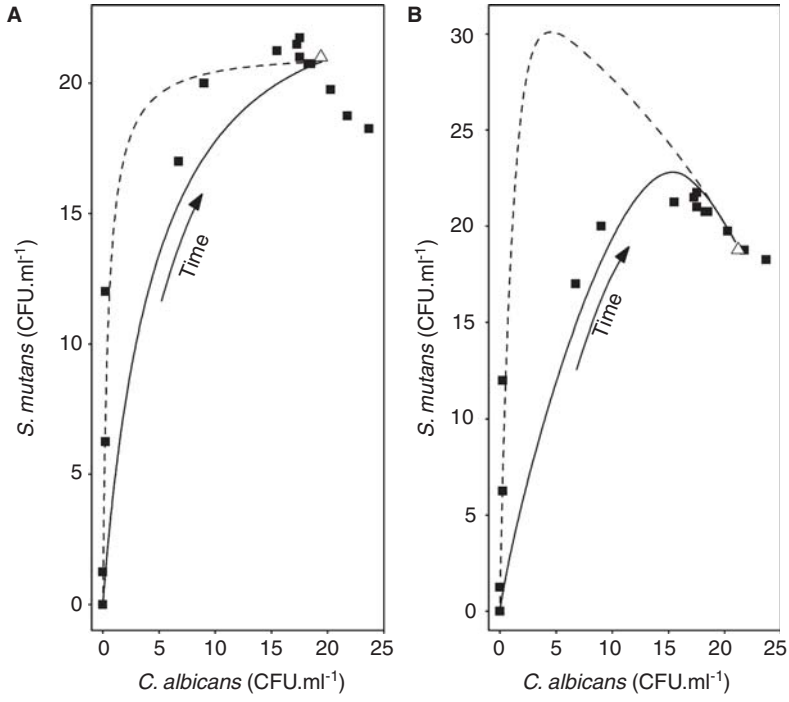

Figure 4. Models trajectories in a states-space representation. Experimental data of *S. mutans* (CFU.ml^{-1}) vs *C. albicans* (CFU.ml^{-1}) when grown together and two simulated trajectories are plotted. Continuous lines show the trajectories obtained with the co-existence model (A) and the competition model (B) using the best fitting parameter values depicted in Table I. Dashed curves depict simulated trajectories obtained using the same parameter values of Table I and different initial conditions ($N_{c/s}{}^0 = 0.1$ and $N_{s/c}{}^0 = 0.05$). White triangles represent the steady state solution for each mathematical model.

Evaluation of the models

Although in general both models are able to describe the growth kinetic for both micro-organisms, the co-existence model—in contrast to the competition model—can only have a monotonic behaviour and, consequently, is not capable of the experimental maximum of *S. mutans* in the presence of *C. albicans*. The previous theoretical considerations were further evaluated using the Akaike criterion (Table I) [14-16] and the residuals analysis [21]. The ΔAIC indicated that both models can be considered as good candidates [16]. On the other hand, the residuals distribution for competition model is random and close to zero independently of the time of incubation for both micro-organisms (insets Figures 3A and B). Contrarily, the co-existence model residuals are characterized by a wider distribution, with its mean further from 0 and a marked bias at long incubation times (insets Figures 2A and B). Consequently, the competition model is theoretically and statistically more adequate to describe our experimental data.

The states space representation allowed us to follow the temporal evolution of one micro-organism with respect to the other in a single graph (Figure 4). The solid lines in Figure 4 show the simulated curves obtained with each model tested here. The even distribution of experimental data around the simulated curve for the competition model (Figure 4B) compared to that of the co-existence model (Figure 4A) strongly suggests that the competition model is more adequate to describe the behaviour of each micro-organism with respect to the other when growing together. In the present experiments, the initial $N(t)$ for *S. mutans* were set to be higher than that of *C. albicans* (see Table I). To evaluate if the initial concentration of each micro-organism (N_i^0) could have an effect on the models response, simulations considering different initial conditions were performed. When N_i^0 for *S. mutans* was augmented 3-times, whilst that for *C. albicans* was diminished in the same factor, the general behaviour of both micro-organisms simulated with the competition model was similar and the temporal maximum was more explicit (Figure 4B, dashed line). As was mentioned before, the co-existence model is not capable to show the maximum (Figure 4A, dashed line).

Finally, to evaluate the consistency of the models we calculated their stationary solutions. Both the co-existence (Figure 4B, white triangle) as well as the competition model (Figure 4A, white triangle) show a clear convergence towards their corresponding steady states. Stability analysis confirmed that these steady states are stable and constitute global attractors (data not shown). The small distance between the experimental data at long incubation times and the stationary solution of competition model indicates that the latest is the only one capable of describing accurately the behaviour of one micro-organism with respect to the other.

Discussion

Numerous studies have focused on the individually growing behaviour of *C. albicans* and *S. mutans* even in the presence of surfaces like orthodontic devices [22–26]. However, experimental reports studying their survival when they share the same niche are scarce [9–11]. Several predictive models have been proposed to take account of growth-influencing factors such as temperature, pH and water activity [27–29]; however, they mostly focus on the growth of single species.

In this report a putative interaction between *C. albicans* and *S. mutans* was studied, experimentally and by mathematical modelling, comparing the growth kinetics of both micro-organisms seeded individually or together during a period of 36 h. To the authors' knowledge, mathematical modelling applied to the interaction between these two micro-organisms was performed for the first time in this study.

Initially, a logistic model of population dynamics [17] was proposed to explain the time course of the number of colonies measured for each species growing separately. The results showed that the logistic model described accurately the behaviour of both micro-organisms when growing individually (Figures 1A and B). When both micro-organisms were grown together, two kinds of models were proposed: the co-existence model, comprising two overlapped logistic models, and the competition model, including a linear competence between both species [20]. Our model validation using the ΔAIC criterion and residual analysis confirmed that the competition model is the best suited to describe the growth curves when both micro-organisms share the niche. Additionally, the states space analysis allowed us to test the consistency of the models and further simulations, varying the initial conditions of our experiments, confirmed the election of the competition model as the best fitting model.

The best fitting parameter values of the competition model when the two species grew together were significantly different from those obtained for the logistic model when the two micro-organisms grew separately (Table I). The initial growth rate (r_i) was higher for each micro-organism when the other was present, therefore *C. albicans* and *S. mutans* are mutually benefited at short times when they grow together. The changes in carrying capacity (K_i) reflect that the final concentration of *C. albicans* is 4-times higher than when grown alone, while *S. mutans* diminished one third its maximal CFU.ml^{-1}. These last changes are probably contributed by the competition mechanism reflected by the α and β parameters. In agreement with these results, it has been previously reported that co-culturing of *C. albicans* and *S. mutans* in oral biofilms on hydroxyapatite increases the growth of *C. albicans* [11]. Additionally, these mutual increases of r_i are in line with the model developed by Dens et al. [30] to study interactions between micro-organisms in food.

By a mechanism known as 'quorum sensing', microbial cells respond to their population density or other external stimulus and regulate gene expression and cellular differentiation [31]. Quorum sensing involves production, secretion and responses to small signal molecules known as autoinducers. Initially thought to be a mechanism only present in bacteria, the discovery of farnesol as a quorum sensing molecule in *C. albicans* [32] demonstrated the existence of quorum sensing in eukaryotic organisms as well. It has been established that *S. mutans* interacts with *C. albicans* through the production of a quorum-sensing molecule, the competence-stimulating peptide [10], as well as through trans-2-decenoic acid [33], while the molecular mechanisms by which *C. albicans* could influence *S. mutants* growth are less clear. Interestingly, depending on environmental conditions and its concentration range, it has been shown that farnesol can increase virulence of *C. albicans* [34]. On the other hand, it has been demonstrated that *S. mutans* biofilm formation is strongly inhibited by farnesol [35]. The farnesol-mediated induction/inhibition of *C. albicans* and *S. mutans* appears as an attractive hypothesis to explain the results observed under our experimental conditions. Further studies at molecular level when both micro-organisms are grown together would be required to confirm this mechanism.

The combined approach of experimental and mathematical modelling used here suggests that during co-colonization a synergistic beneficial process operates between *S. mutans* and *C. albicans* at short times, followed by a competition at longer times where *S. mutans* is finally outcompeted by *C. albicans*. It has been demonstrated that early colonizers may promote the establishment of other species that become more dominant in dental plaque as it develops [36]. According to our results it could be argued that prior infection of the oral cavity with *S. mutans* may facilitate and even promote colonization by *C. albicans*. On this basis, it appears attractive to further identify the precise biological factors regulating the interaction between these two species in order to completely elucidate the underlying mechanism.

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