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Modelling growth/no growth interface of *Zygosaccharomyces bailii* in simulated acid sauces as a function of natamycin, xanthan gum and sodium chloride concentrations

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Abstract

Probabilistic microbial modelling using logistic regression was used to predict the growth/no growth (G/NG) interfaces of *Zygosaccharomyces bailii* in simulated acid sauces as a function of natamycin, xanthan gum (XG) and sodium chloride concentrations. The growth was assessed colorimetrically by using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride and 2-methoxy-1,4-naphthoquinone as detection reagents. The logistic regression model successfully predicted G/NG probability. The detection reagents used allowed the evaluation of G/NG interfaces in opaque systems with an excellent agreement with the plate count method. Natamycin concentration of 12 mg/L was needed to inhibit *Z. bailii* growth independently of the presence of XG and/or NaCl. Addition of 3.00 and 6.00% of NaCl exerted an antagonistic effect on natamycin action. Furthermore, addition of 0.25 and 0.50% XG decreased natamycin and/or NaCl action. However, an increased in XG concentration to 1.00% decreased yeast growth. Mentioned results highlighted the importance of the correct selection of stress factors applied to inhibit *Z. bailii* growth.

Keywords: *model acid sauces; growth/no growth interface; natamycin; xanthan gum; sodium chloride; Zygosaccharomyces bailii.*

Abbreviations: G/NG, growth/no growth; XG, xanthan gum; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride; MNQ, 2-methoxy-1,4-naphtho quinone; SB, Sabouraud broth; SA, Sabouraud agar.

1. Introduction

In recent decades, the nutritional and economic importance of acid sauces has increased, because they are used in the food industry to improve the attractiveness and flavor of food. Acid sauces are liquid or semi-solid dispersed systems whose structure is provided by thickening, gelling and/or emulsifier agents. They include concentrated suspensions and salad dressings (Mertens et al., 2009). Acid sauces are

shelf stable foods. This condition is achieved by depressing pH, water activity and by the addition of preservatives (Vermeulen et al., 2007a). However, they are prone to be spoiled by acid resistant yeasts (Monu et al., 2016). In particular, *Zygosaccharomyces bailii,* was isolated from salads and mayonnaise dressings (Couto et al., 1996). This yeast is resistant to adverse environmental conditions. It can grow in products with a pH of 3.6 and a water activity of 0.89 (Smittle, 2000).

Because of the consumer demand for “preservative-free” and “natural” food products, interest has increased in the food industry for natural antimicrobials to maintain microbiological control in foods (Monu et al., 2016).

Natamycin is a natural fungicide produced by *Streptomyces natalensis*, which is commonly used in dairy food products to control spoilage by fungi and yeasts (El-Diasty et al., 2008).The antifungal activity of natamycin depend on the substancer binding to the cell membrane sterols, mainly ergosterol, which is the main sterol of fungal membranes, which makes them permeable (Pedersen, 1992). Furthermore, the activity is modified by the presence of other stress factors and additives and this issue has not yet been studied in detail (Arroyo-Lopez et al., 2012). It must be stressed that the effect of thickening and/or emulsifier agents on microbial growth and on preservatives activity is controversial (Campos et al., 2015). Furthemore, it has not been evaluated in relation to natamycin activity.

Determination of microbial G/NG interfaces is a useful tool to evaluate microbiological stability and antimicrobial effectiveness. The G/NG boundary of microorganisms can be examined by probabilistic models as a function of the stress factors applied (Presser et al., 1998; Ratkowsky and Ross, 1995). The combination of stress factors that assure low probability of growth is a key factor to determine product formulation (Dang et al., 2010). Growth/no growth models were previously developed for *Z. bailii*. Jenkins et al. (2000) studied the G/GN interfaces of *Z. bailli* as a function of pH, NaCl, fructose and acetic acid in acidified products, by using models for time to growth and probability of growth. López-Malo and Palou (2000) also modelled the

probability of growth of *Z. bailli* as a function of water activity, temperature, sodium benzoate and potassium sorbate in mango puree. Dang et al. (2010) modelled the probability of growth of *Z. bailli* as a function of water activity, pH, acetic acid in acidified sauces. However, all mentioned studies were done in liquid media and therefore, the effect of structure was not considered. Previously, the effect of XG on *Z. bailii* growth in model systems resembling acid sauces it was evaluated. Results obtained showed that the structure promoted by the gum exerted a significant effect on growth (Zalazar et al.,2016).

The main goal of the present study was to obtain the G/NG interfaces of *Z. bailii* for natamycin in combination with NaCl and XG, which was accomplished by means of logistic modelling. We also evaluated the colorimetric method to assay cell yeast growth using 2- (4-iodophenyl) -3- (4-nitrophenyl) -5-phenyl-2H-tetrazolium chloride and 2-methoxy-1,4-naphthoquinone as detection reagents. The results obtained in this study will be used to assess a possible application of this antimycotic as a preservative agent in acid sauces.

2. Materials and Methods

2.1. Materials

2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), 2-methoxy-1,4-naphthoquinone (MNQ) were obtained from Sigma Aldrich, USA. Reagent grade citric acid and sodium chloride were from Anedra (Argentina). Food grade xanthan gum was from Cargill (Argentina) and natamycin was from Handary S.A. (Belgium). All culture media used for microbiological evaluations (SB, Sabouraud broth; SA, Sabouraud agar; peptone water; glycerol) were from Biokar (Biokar Diagnostics, Beauvais, France).

2.2. Yeast strain, inocula preparation and viability determination

Zygosaccharomyces bailii NRRL 7256 was stored at -80.0 ± 1.0 ºC in SB broth plus 10% w/w glycerol. Before its use, the strain was grown twice in SB at 25.0 ± 0.5 ºC for 24 h.Then the tubes were centrifuged at 7500 g for 10 min and the pellet re-suspended into peptone water. Thus, yeast cultures were obtained with more than 10^8 CFU/ml. After that, the inoculum was diluted in peptone water to reach 0.5 McFarland units, corresponding to a population of approximately 10^6 CFU/ml. Turbidity was measured with a turbidimeter (Densichek Biomerieux, France).

2.3. Preparation of detection reagent

Tetrazolium salt INT was dissolved in distilled water at 0.140%w/v. Electron mediator, MNQ, was dissolved in dimethylsulfoxide at 0.020%w/v. Both solutions were sterilized by membrane filtration. They were combined at a ratio of 9:1INT-MNQ.This detection reagent prepared contained 0.126%w/v of INT and 0.002%w/v of MNQ (named INT-MNQ) and mentioned concentrations were selected taking into account results from previous studies (Zalazar et al., 2018, a).

2.4. Model system preparation

Model systems were formulated in SB with the addition of different concentrations of natamycin, NaCl and XG. All the ingredients, were suspended in distillated water and poured into glass flasks.The XG was dispersed by agitation for 24 h at 25ºC to assure complete hydratation. Systems pH was adjusted to 3.50 with citric acid solution. The systems were thermally treated for 15 min at 95°C to avoid antimicrobial degradation and they were dispensed in sterile 48-well flat-bottom microplates (Kartell, Italy). Aliquots of 850 µl each were placed in quadruplicate and 100 µl of an inoculum of 10^5 CFU/ml of *Z. bailii* was added. Uninoculated wells for each system without adjusted pH and inoculated system without adjusted pH were also included as controls.

The microplates were incubated 100 h at 25 ± 0.5°C.This time was selected to ensure growth inhibition since in previous studies it was found out that natamycin extended yeasts lag phase (Zalazar et al., 2018, a). Each microplate was made in duplicate and some systems that presented unexpected results were performed in quadruplicate. Thus, between 8 and 16 repetitions were obtained per system.

2.5.Yeast growth evaluation

The effect of natamycin, NaCl and XG concentrations on *Z. bailii* on G/NG boundary was determined by a colorimetric method previously developed and based on the reduction of INT and MNQ as detection reagents. For this purpose, at the end of incubation, 50 µl of INT-MNQ mixture was added and the microplate was incubated for 2 h at 25°C. The visual detection of indicator color change in the wells, as compared with the negative and positive controls, was considered as absence of inhibition. Furthermore, yeast viability at interfaces was determined by surface plating on SA. To perform this, some wells of each system were kept without the addition of the redox indicator. Colonies were counted after 5 days at 25°C.

2.6. Experimental design and data analysis

A full-factorial design with 96 treatments resulting from the combination of 6 levels of natamycin (0, 2, 4, 6, 10 and 12 mg/L), 4 levels of NaCl (0.00, 1.50, 3.00 and 6.00% w/w) and 4 levels of XG (0.00, 0.25, 0.50 and 1.00%w/w), was used in the present study. Each system was replicated from 8 to 16 times. Thus, a total of 976 data were obtained. Statistical data analysis were performed with R version 3.4.2 R (Core Team, 2017, Vienna, Austria).

2.7. Development of growth/no growth interfaces by logistic regression modelling

From G/NG data obtained according to the turn of the indicator, a binary variable was created. Then, a logistic regression model of this variable was adjusted as a function of system component concentrations. The equation of the ordinary logistic regression model consists of a polynomial (right-hand side) and $\text{logit}(p) = \ln\frac{p}{1-p}$ (left-hand side) with p the probability that growth occurred (p takes values between 0 and 1) (Vermeulen et al., 2007a, 2007b). A threshold value for the final development of the yeast was established: if the number of CFU/ml exceeded the threshold necessary for the turn of the indicator, it was considered that there was growth (coded as "1"), and if it was below - no change in the indicator was observed - there was no growth (coded as "0"). The logistic regression model selected included the main factors, their interactions and the quadratic expression of main factors. The model is shown in Equation 1:

$$logit(p) = b_0 + b_1.x_1 + b_2.x_2 + b_3.x_3. + b_4.x_1^2 + b_5.x_2^2 + b_6.x_3^2 + b_{12}.x_1.x_2 + b_{13}.x_1.x_3 + b_{23}.x_2.x_3 \quad (1)$$

Where x_1 ,x_2 and x_3 are the concentrations of natamycin, XG and NaCl, respectively; b_0, is an intercept; b_1, b_2 and b_3 are the coefficients of the linear terms; b_4, b_5 and b_6 are the coefficients of the quadratic terms; and b_{12}, b_{13} and b_{23} are the coefficients of the interactions.

The G/NG interfaces for *Z. bailii* can be deduced from Equation 1. The quality of the fitted model was assessed with several tests. First, the deviances of the model were determined. Secondly, the value of the Akaike's Information Criterion (AIC) and chi-square likelihood ratio were used to compare the model with a previous one which only considered the linear form of factors and interactions.

The predictive power of the model was measured through the estimation of the area under the Receiver Operating Curve (ROC). The maximum value is 1 and

indicates a perfect predictive power and a value of 0.5 indicates a that the predictions are not better than random.

A cross-validation technique with ten iterations (*10-fold cross-validation)* was used to validate the predictive quality of the model. This technique consists in dividing the data set into tenths at random and taking nine tenths of the data (training data) to fit the model. Then, the tenth that was left out (test data) is used to make predictions with the model and compare those predictions with the real data. This procedure is iterated nine more times, until all combinations of training and test data were analyzed. Then, a matrix of confusion was assembled with the observed data of G/NG and those predicted by the model and the error was calculated as the sum of the number of cases that are located on the main diagonal divided by the total. In addition, the specificity and sensitivity were calculated, which are defined as follows:

$$\text{Sensitivity} = TP/(TP + FN) \quad (2)$$

$$\text{Specificity} = TN/(TN + FP) \quad (3)$$

where *FN* (false negatives) are the cases where there was growth but the model classified them as no growth; *FP* (false positives) are the cases where there was no growth, but they were classified as growth; *TP* are the cases correctly classified as true positives and *TN*, the true negatives. Therefore, sensitivity measures the ability of the model to detect cases with growth, and specificity measures the ability to "not be confused" with false positives. Finally, after carrying out the cross-validation, mean error, sensitivity and specificity were calculated, considering probability cut-off points of 0.3; 0.5 and 0.7, to know if the model predicted a growth probability greater than 0.3, 0.5 or 0.7, respectively.

3. Results and discussion

3.1. Description of trends observed in growth/no growth data

The total number of treatments analyzed in the present work were 976 and the distribution of the G/NG data was 53/36 and in seven systems both trends (G and NG) were observed, which indicated that the conditions tested globally favored growth. Of the total number of treatments, 24 were carried out in broth, and the distribution of the G/NG data was 16/7 and in one system both trends were observed. This indicated that in liquid medium there was a greater proportion of cases of no growth. On the other hand, the rest of the systens 72) corresponded to systems structured with XG, and the distribution of the G/NG data was 46/20 and in six systems both trends were observed.These results showed that the presence of XG favored the growth of the yeast. In addition, it is necessary to differentiate the growth in liquid from a more structured medium since the yeasts grow in different ways.

Most models have been developed in liquid medium, which can successfully mimic the microbial growth environment of liquid foods or dispersed systems with low viscosity, where the microbial growth site is in the aqueous phase (McKellar and Lu, 2001; Presser et al., 1998; Salter et al., 2000; Tienungoon et al., 2000). In these cases, the nutrients and metabolites are uniformly distributed in the medium, and the growth of microorganisms is planktonic. However, in solid or semi-solid foods, microorganisms can also grow on the surfaces or within the substrate. In these conditions, microorganisms are immobilized and forced to form colonies (Koutsoumanis et al., 2004). In the studied systems, visual observation showed that *Z. bailli* grew as colonies when XG was 0.50 or 1.00% (Fig.1, Zalazar et al., 2018, b).

As mentioned previously, the presence/absence of growth was determined by the color change of detection reagent (Fig.2, Zalazar et al., 2018, b). Furthermore, growth was confirmed by plate count. In all cases, the assumption was satisfactorily confirmed by both methodologies. As an example, the results of two microplates are shown in Fig.1, in which the growth of *Z. bailii* was manifested by the appearance of color in the wells. In the absence of NaCl (Fig. 1, Panel A), growth was observed in all

systems, except in those containing 6 mg/Lppm of natamycin. In addition, it can be observed that the addition of XG intensified the color. When adding 3.00% of NaCl, growth was inhibited in all systems without XG (Fig.1, Panel B), but growth was observed in the presence of gum. In the presence of 3.00% NaCl, growth is also inhibited in systems with 6 mg/L natamycin. Table 1 illustrates the concordance between detection reagent and plate count for selected cases and more examples can be found in Table 2 of Zalazar et al. (2018, b).

The effect of the three factors studied will be analyzed in the next sections.

3.1.1. Effect of natamycin

The antimicrobial effect of natamycin appeared after 6 mg/Lwhere growth was recorded in some cases and not in others depending on system composition (Fig. 2, Panel A-D). The addition of 0.25% XG in the NaCl free system decreased the antimicrobial effect exerted by 6 mg/L. However, as the concentration of XG increased, this trend reversed (Fig. 2, Panel D). The later was also observed for the system with 10 mg/L natamycin but in the presence of 3.00% NaCl (Fig.2, Panel E). On the other hand, the increase in NaCl concentration in these systems also decreased the antimicrobial effect, suggesting the existance of an antagonistic effect. These trends showed that there is an interaction between natamycin, XG and NaCl. Although the inhibitory effect of natamycin in yeast and molds is widely known (Thomas and Delves-Broughton, 2003), the interaction with different environmental factors has not yet been studied in detail. In particular, there is no data about the influence of stabilizing agents. Finally, the highest level of natamycin (12 mg/L) inhibited the growth of *Z. bailii* independently of the presence of XG and/or NaCl (Fig.2, Panel F). In agreement with these results, Gallo et al. (2006) reported that 12.5 mg/L was necessary to inhibit the growth of *Sacharomyces cerevisiae* in cheese whey and Arroyo-López et al. (2012)

found that 10-22 mg/L was needed to inhibit *S. cerevisiae*, *Candida boidinii* and *Wickerhamomyces anomalus* in liquid culture medium with 4.50% NaCl.

3.1.2. Effect of NaCl

The use of 1.5% NaCl did not modify the level of natamycin necessary to inhibit yeast growth, which was 10 mg/L, regardless of XG concentration (Fig.3, Panel B). However, it increased the growth probability from 50% to 100% in systems containing natamycin (6 mg/L) free of XG and with 0.50% XG (Fig.3, Panel A vs B).

Increasing NaCl level to 3.00% or 6.00% inhibited growth in all systems without XG, regardless of natamycin presence (Fig.3, Panels C and D). However, the inhibitory action of NaCl on *Z. bailii* was nullified by the adittion of XG. These results suggested the existence of an antagonistic action between XG and NaCl. Boons et al. (2013) observed a decreased in *SalmonellaTyphimurium* and *Escherichia coli* growth lag phase when XG or gelatin were added to a medium containing salt and they suggested that NaCl could interact with these structuring agents and as a consequence lower concentrations of NaCl would be detected by the cells. Furthermore, XG by itself can influence yeast growth as it will be discussed in the next section.

On the other hand, in the presence of 0.25 or 0.50%XG, the increase in NaCl concentration to 3.00 or 6.00% decreased the antimicrobial effect of natamycin. As a consequence the amount of the antimicrobial to inhibit growth increased from 10 to 12 mg/L as NaCl increased from 1.50 to 6.00% (Fig. 3, Panel B vs Panels C and D). A similar trend was observed for *Saccharomyces. cerevisiae* and *C. boidinii* incubated in the presence of 5.00% NaCl (Arroyo Lopez et al., 2012). Probably, this antagonistic action is related to the reduction in ergosterol content promoted by the presence of NaCl as it was verified in *S. cerevisiae* cells, where ergosterol decreased a 40% in response to stress caused by 5.84% of NaCl (Montañés et al., 2011).

3.1.3. Effect of xanthan gum

The addition of 0.25% or 0.50% XG promoted yeast growth in systems with 3.00 and 6.00% NaCl. This raised the concentration of natamycin needed to achieve inhibition to 12 mg/L (Fig.4, Panel A vs Panels B and C). This antagonistic effect can be attributed to the promoting action on growth exerted by XG since it was found that this gum can be use as carbon source as it was demonstrated in a previous study (Zalazar et al.,2016).

It is noteworthy that, by increasing the concentration of XG to 1.00%, the necessary amount of antimicrobial to inhibit yeast growth was reduced to 10 mg/L, regardless of NaCl presence (Fig.4, Panel D). This effect can be linked to the structuring effect of the medium provided by the higher level of XG. A concentration of 1.00% modifies the rheology of the system from a weak gel to a strong gel as it was revealed by the mechanical spectra of suspensions containing different concentrations of the gum (Zalazar *et al.,* 2016). Therefore, the presence of 0.25% and 0.50% XG favors growth, since at these levels the gum is also a nutrient, but when the concentration is increased to 1.00%, its structuring effect prevails. This effect can slow the growth of microorganisms, which develop in the form of colonies.

Several authors investigated the effect of food structure and cell immobilization on microbial behavior and showed that microorganisms limited to growth as colonies show a different metabolic activity and a slower growth rate compared to planktonic growth (Brocklehurstet al., 1997; Meldrum et al., 2003; Skandamis et al., 2000; Stechiniet al., 1998; Wilson et al., 2002). The difference between the growth limits of immobilized and planktonic cells can be attributed to factors such as cell physiology, nutrient diffusion, oxygen availability or cell-to-cell communication (Fuqua et al., 1994; Koutsoumanis et al., 2004; Stechini et al., 1998; Walker et al., 1998; Yoon et al., 2003). Moreover, system structure, provided for example for a gelling agent also modified microbial growth cinetic parameters (Skandamis and Jeanson 2015).

In summary, XG affected negatively the action of NaCl and natamycin and this effect depended on the concentration of the gum.

3.2. Modeling of growth/no growth interfaces by logistic regression

Growth/no growth data were adjusted to a logistic regression model that consider linear and quadratic terms of the design factors (concentration of NaCl, natamycin and XG) and their interactions. The estimated coefficients and statistic parameters of the model are shown in Table 2. It can be observed that all the factors were significant, either in their linear or quadratic form, or through some interaction. The effect of XG was significant, both in its linear and quadratic form. In addition, two significant interactions were obtained: natamycin-XG and natamycin-NaCl. Furthermore, the residuals were smaller than those of the null model (null model deviance = 160.4 over 122 degrees of freedom and the residual deviance of the adjusted model = 58.6 over 113 degrees of freedom). The adjustment test of the residuals to a chi square distribution also suggested that it can be accepted that the residuals have such a distribution (P = 0.999). The AIC value was 78.587, which was lower than the one of the model that only considered the linear form of parameters and interaction (AIC = 97.055). This demonstrated that the inclusion of the quadratic term gave a greater explanatory value to the model. A comparison between both models with an analysis of variance by using a chi square test confirmed that the models have different explanatory power ($P = 2x10^{-5}$).

Figure 5 shows the exploratory graphs of the relationships between natamycin, NaCl and XG that present the curves predicted by the model. In general, antimicrobial inhibitory action was observed from a concentration of 6 mg/L, where the fall in the growth probability curves was observed. In the absence of XG, the antimicrobial inhibitory effect depended on NaCl concentrations: at low salt levels, the curves showed a drop at 6 mg/Lof natamycin (Panels 1.A and 1.B) but, at higher levels of NaCl (3.00 and 6.00%), the shape of the curves changed, decreasing the probability of

growth and becoming less dependent on the level of natamycin (Panels 1.C and 1.D). On the other hand, in the presence of 0.25 and 0.50% XG the inhibitory effect of natamycin decreased (Panels 2.A-D and 3.A-D), but the increase to 1.00% XG reversed this trend (Panels 4.A to 4.D), thus the interaction between natamycin and XG was verified. In addition, the presence of 3.00 and 6.00% NaCl also had a negative effect on antimicrobial activity, especially at concentrations of 0.25 and 0.50% XG (Panels 2.C-D and 3.C-D). These results are an expression of the interaction of natamycin with NaCl previously commented. Similar trends were reported by Arroyo-López et al. (2012), who observed that at concentrations close to 5.00% of NaCl the resistance of *S. cerevisiae* and *C. boidinii* to natamycin increased slightly, although concentrations above 6.00% of NaCl favored the inhibition exerted by this antifungal. Furthermore, it was observed that at low concentrations of NaCl (0.00-1.50%), XG did not influence the effect of natamycin on the probability of growth, with the exception of the level of 6mg/L, where the increase in XG decreased the antimicrobial effect (Panels 1-4.A and 1-4.B). However, when the concentration of NaCl was increased (Panels 1-4.C and 1-4.D) the presence of XG influenced the probability of growth of the yeast, therefore their interaction was manifested. In previous studies, different trends were observed regarding the effect of NaCl in structured media. For example, Mertens et al. (2011) studied the G/NG probability of *Z. bailii* in systems containing 7.5% NaCl, acidified with acetic acid at pH 3.5 and stored at 30°C. These authors reported a growth probability of 100% both in the absence and in the presence of 2.5% XG. However, in the same test conducted at 22°C, the probability of growth was greater in the structured medium than in the liquid medium. These trends indicate that the probability of growth depends on different factors besides the structure as it was previously mentioned.

At low levels of natamycin, high levels of NaCl and in the absence of XG, the probability of growth was low, but the addition of the gum reversed this trend (Fig. 5

Panels 1-4C and 1-4D). However, at higher levels of natamycin (6 and 10 mg/L), the increase in XG reduced the probability of growth. Nevertheless, 12 mg/L of natamycin reduced the probability of growth, independently of the presence of the other factors. At this antimicrobial concentration, the system enters in a regime where, regardless of the concentration of the other factors, inhibition is obtained.

In general, the curves predicted by the model correctly adjusted the data obtained, except in treatments with 3.00 and 6.00% NaCl, in the absence or presence of 0.25% XG (Panels 1.C and 2.C-D), where the model predicted growth but no growth data were obtained or vice versa. A similar trend was observed by Arroyo-López et al. (2012), when studying the G/NG interfaces of several deteriorating yeasts. Furthermore, unexpected changes in the probability of growth often occurred around the transition zones (between growth and no growth), indicating that small variations in environmental conditions near the G/NG limits can cause a significant impact in the probability of growth (Dang et al., 2010).

The area under ROC was 0.923 which sugested that the predictive power of the model was good.

The cross-validation technique and the confusion matrix were used to validate the model. The confusion matrix showed that there were 12 cases where growth was observed, and the model also predicted growth, and 3 cases where there was no growth and the model predicted growth. On the other hand, there were 4 cases where growth was observed, and the model predicted no growth, and 10 cases where there was no growth and the model also predicted no growth.

Table 3 shows the media errors sensitivity and specificity obtained considering probability cutoff points of 0.3, 0.5 and 0.7. It can be seen that the error is a bit high and similar (between 13-15%) for the three cut points. This error is mainly due to the combinations of 6 mg/Lof natamycin with the highest levels of NaCl (3.00-6.00%) and XG (0.50-1.00%) where the model presented a lack of fit. Therefore, the choice of the

cut-off point, was based on sensitivity and specificity values. The probability cut-off point of 0.3 showed greater sensitivity (0.969) but lower specificity (0.731), which means that it has a greater ability to detect cases with growth and lower ability to "not be confused" with false positives, that is, cases in which there was no growth but the model predicted them as growth. On the other hand, the probability cut point of 0.7 presented lower sensitivity (0.837) but greater specificity (0.917), which means that it has less ability to detect cases with growth but a greater ability to avoid false positives. A very specific test is chosen when the cost of obtaining false positives is preferred to the cost of false negatives (cases in which there was growth but the model predicted them as not growth). In contrast, a very sensitive test is chosen when it is preferred to obtain false positives instead of false negatives, that is, the number of undetected growths is required to be minimal. Therefore, a probability cutoff point of 0.3 was selected, to avoid spoiling conditions.

4. Conclusions

Results commented demonstrated that the use of the redox indicator allowed the evaluation of G/NG interfaces in opaque systems with an excellent agreement with the plate count method.

Z. bailii growth was influenced by the presence of natamycin, NaCl and XG in systems modeling acid sauces.

Natamycin concentration of 12 mg/Lwas needed to ensure growth inhibition of *Z. bailii* independently of the presence of XG and/or NaCl. On the other hand, the addition of 3.00 and 6.00% of NaCl exerted an antagonistic effect on the action of 6 and 10 mg/Lof natamycin in the presence of 0.25 and 0.50% XG, which was attributed to a possible reduction in the ergosterol content of the yeast caused by stress by NaCl presence.

Xanthan gum influenced the growth of the yeast independently of natamycin and/or NaCl presence. In the absence of XG, the effect of natamycin and NaCl was

remarkable, but the addition of 0.25 and 0.50% XG decreased the antimicrobial effect of natamycin and/or NaCl, which was attributed to its effect as nutrient. However, increasing the gum concentration to 1.00% decreased the growth, which was related to structurant effect of this concentration. This trend stressed that system structure is a key factor that must be taken into account when modeling the limits of microbial growth.

The logistic regression model used successfully predicted yeast G/NG probability as a function of natamycin, NaCl and XG, giving a good estimation of the responses in the range studied. Moreover, results highlighted the importance of the correct selection of the stress factors to be applied to inhibit the development of *Z. bailii*, the main deteriorating yeast of acid sauces. The G/NG interface models are valuable tools for describing the conditions, which can be applied to control a process or design a product formulation for a safer food processing.

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Figure 1. Determination of *Z. bailii* growth/no growth interfaces after 3 hours of adding the redox indicator in the absence of NaCl (Panel A) and in the presence of 3.00% NaCl (Panel B). *Columns 4 and 8 without redox indicator (kept for plate count).*

Figure 2. Growth/no growth interfaces of *Z. bailii* as a function of NaCl and XG concentrations in the presence of different levels of natamycin.

Panel A: 0 mg/L of natamycin; Panel B: 2 mg/L of natamycin; Panel C: 4 mg/L of natamycin; Panel D: 6 mg/L of natamycin; Panel E: 10 mg/L of natamycin and Panel F: 12 mg/L of

natamycin. Symbols: (■): 100% of growth; (O): 0% of growth and (△): growth between 0 and 100% (with the indicated percentage).

Figure 3. Growth/no growth interfaces of *Z. bailii* as a natamycin and XG concentrations in the presence of different levels of NaCl.

Panel A: 0.00% NaCl; Panel B: 1.50% NaCl; Panel C: 3.00% NaCl and Panel D: 6.00% NaCl. Symbols: (■): 100% of growth; (O): 0% of growth and (△): growth between 0 and 100% (with the indicated percentage).

Figure 4. Growth/no growth interfaces of *Z. bailii* as a function of natamycin and NaCl concentrations in the presence of different levels of XG.

Panel A: 0.00% de XG; Panel B: 0.25% de XG; Panel C: 0.50% de XG and Panel D: 1.00% de XG. Symbols: (■): 100% of growth; (O): 0% of growth y (△): growth between 0 and 100% (with the indicated percentage).

Figure 5. Relationship between the variables (XG, NaCl and natamycin) and the curves predicted by the model as a function of natamycin concentrations.

Table 1. *Z bailii* counts at interfaces

Well - System	***Z. bailii* population** (log CFU/ml)± standard deviation)
B8 (Fig 1 A) - 4 mg/L natamycin/ 0.50% GX/ 0.00% NaCl	6.92 ± 0,05
E8 (Fig 1 A) - 6 mg/L natamycin/ 0.50% GX/ 0.00% NaCl	3,20± 0,03
A4 (Fig 1 B) - 0 mg/Lnatamycin/ 0.00 % GX/ 3.00% NaCl	3,70 ± 0,06
B4 (Fig 1 B) - 0mg/L natamycin/ 0.50% GX/ 3.00% NaCl	8,09± 0,04

Table 2. Coefficients, standard errors and statistics obtained from the second logistic regression model.

Parameter	Estimated coefficient ± Standard error	Z value	P (< | z |)
Interception(b_o)	3.962 ± 1.751	2.262	**0.023673**
Natamycin(b_1)	-0.267 ± 0.413	-0.648	0.51682
Xanthan(b_2)	24.709 ± 7.810	3.164	**0.001558**
NaCl(b_3)	-1.344 ± 0.706	-1.903	0.057100
Natamycin^2(b_4)	-0.050 ± 0.034	-1.472	0.140999
Xanthan^2(b_5)	-17.274 ± 5.067	-3.409	**0.000651**
NaCl^2(b_6)	-0.029 ± 0.091	-0.316	0.752361
Natamycin: Xanthan(b_{12})	-1.486 ± 0.676	-2.194	**0.028266**
Natamycin: NaCl(b_{13})	0.189 ± 0.062	3.023	**0.002506**
Xanthan: NaCl(b_{23})	0.674 ± 0.438	1.537	0.124296

The Z-value shows the value of the statistics used to assess the significance of the estimated coefficient and P (< | z |) shows the probability of observing the absolute value of that Z-value under the null hypothesis of no effect of the factor or interaction. When this probability was less than 0.05, the effect was considered significant.

Table 3. Averages of errors, sensitivity and specificity at probability cut points of 0.3, 0.5 and 0.7

Cut point	Error	Sensitivity	Specificity
0.3	0.133	0.969	0.731
0.5	0.167	0.897	0.764
0.7	0.150	0.837	0.917

Highlights

- Logistic regression model predicted G/NG *Z. bailii* probability in model acid sauces
- Effect of natamycin, NaCl and xantham gum levels on G/NG interfaces was evaluated
- NaCl (3.00 or 6.00 %) exerted an antagonistic effect on natamycin action
- XG (0.25 or 0.5 %) exerted an antagonistic effect on NaCl and natamycin action
- XG (1.00 %) decreased yeast growth and improved natamycin action

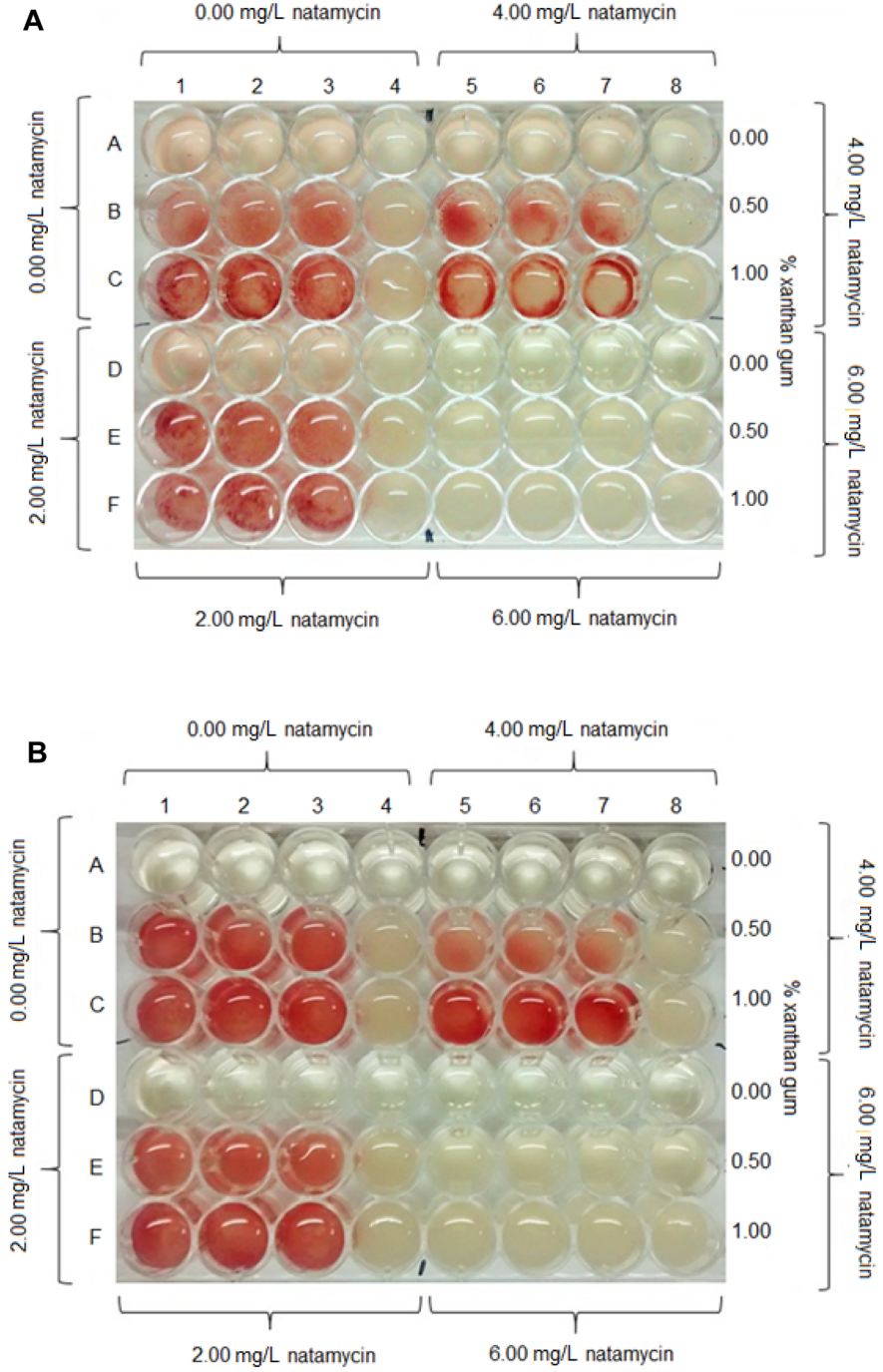
A
0.00 mg/L natamycin
4.00 mg/L natamycin
1 2 3 4 5 6 7 8
0.00 mg/L natamycin
2.00 mg/L natamycin
A
B
C
D
E
F
0.00
0.50
1.00
0.00
0.50
1.00
% xanthan gum
4.00 mg/L natamycin
6.00 mg/L natamycin
2.00 mg/L natamycin
6.00 mg/L natamycin
B
0.00 mg/L natamycin
4.00 mg/L natamycin
1 2 3 4 5 6 7 8
0.00 mg/L natamycin
2.00 mg/L natamycin
A
B
C
D
E
F
0.00
0.50
1.00
0.00
0.50
1.00
% xanthan gum
4.00 mg/L natamycin
6.00 mg/L natamycin
2.00 mg/L natamycin
6.00 mg/L natamycin

Figure 1

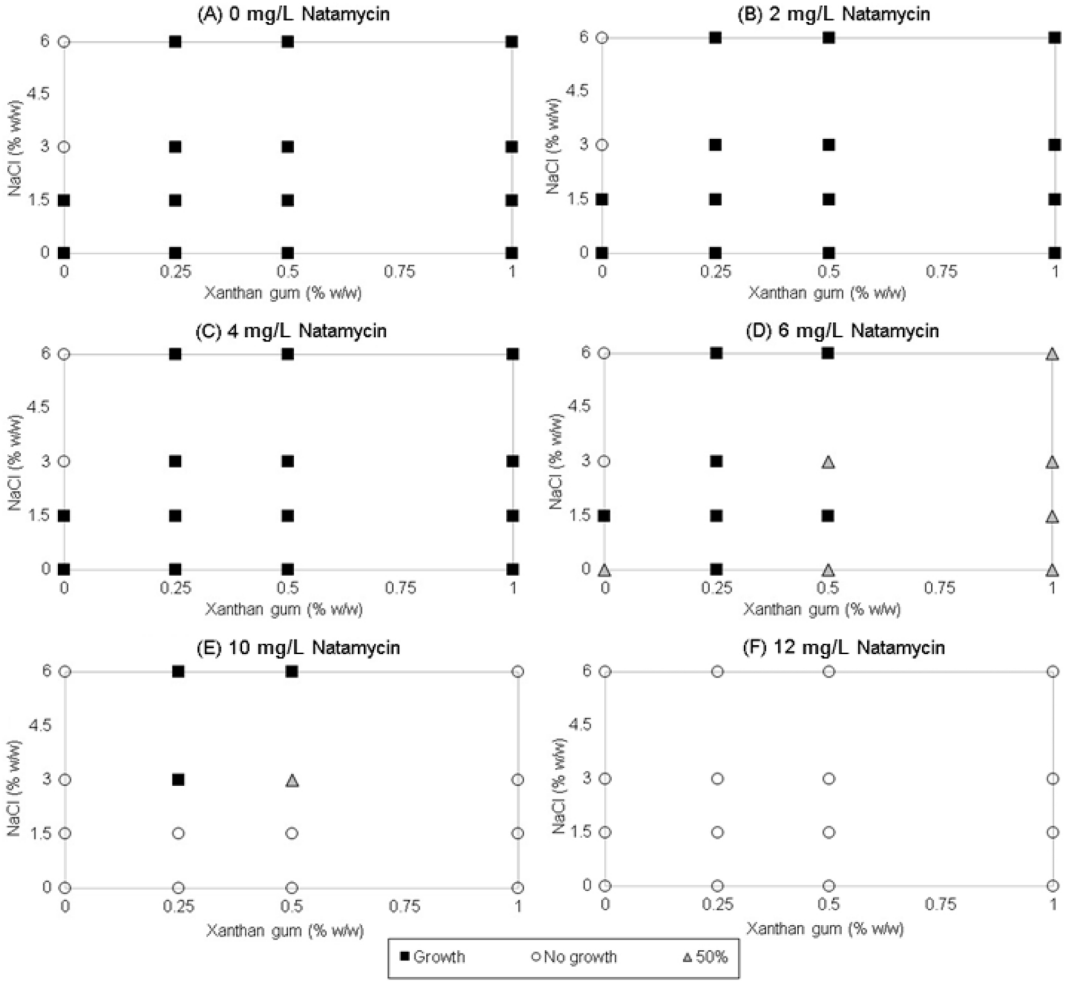
(A) 0 mg/L Natamycin
(B) 2 mg/L Natamycin
(C) 4 mg/L Natamycin
(D) 6 mg/L Natamycin
(E) 10 mg/L Natamycin
(F) 12 mg/L Natamycin
NaCl (% w/w)
Xanthan gum (% w/w)
0
1.5
3
4.5
6
0.25
0.5
0.75
1
Growth
No growth
50%

Figure 2

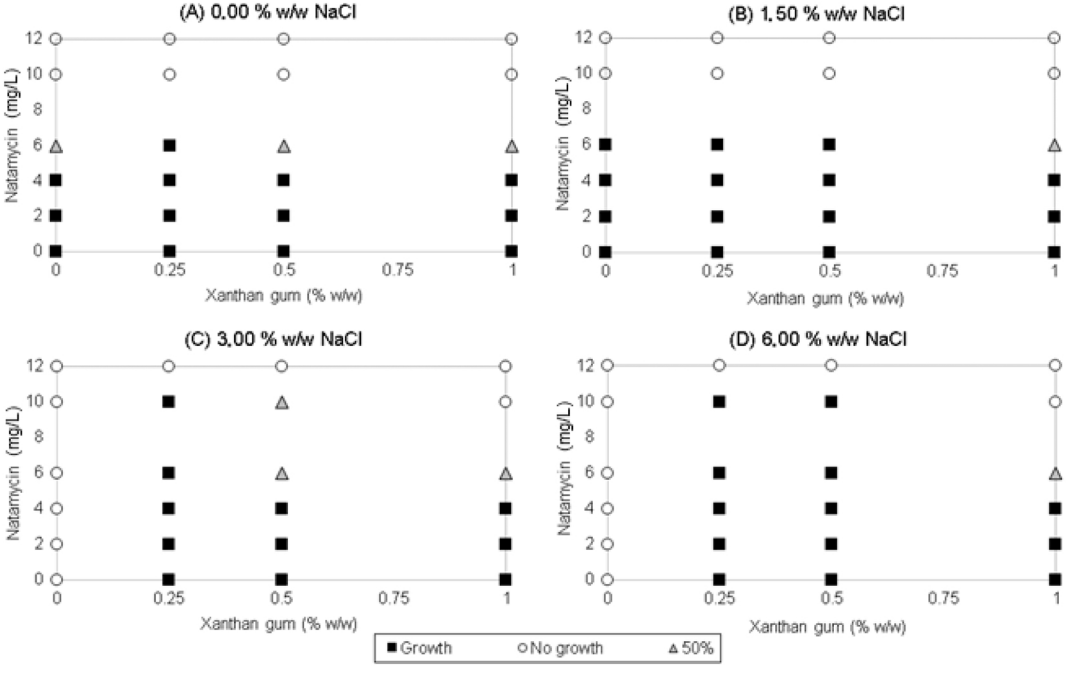
(A) 0.00 % w/w NaCl
(B) 1.50 % w/w NaCl
(C) 3.00 % w/w NaCl
(D) 6.00 % w/w NaCl
Natamycin (mg/L)
Xanthan gum (% w/w)
0
2
4
6
8
10
12
0.25
0.5
0.75
1
Growth
No growth
50%

Figure 3

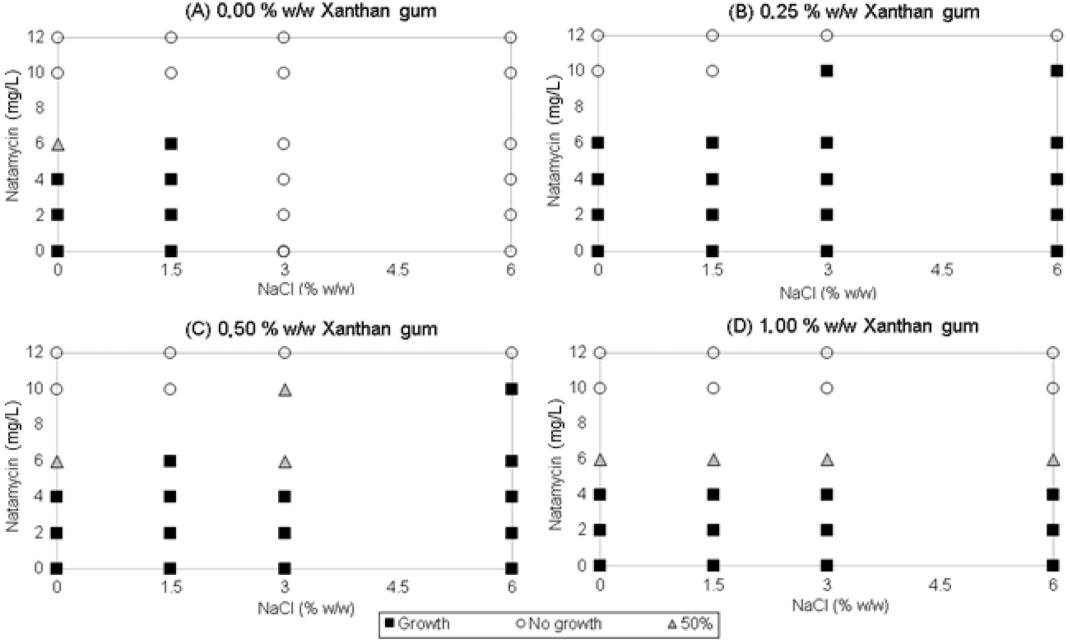
(A) 0,00 % w/w Xanthan gum
(B) 0,25 % w/w Xanthan gum
(C) 0,50 % w/w Xanthan gum
(D) 1.00 % w/w Xanthan gum
Natamycin (mg/L)
NaCl (% w/w)
0
1.5
3
4.5
6
0
2
4
6
8
10
12
Growth
No growth
50%

Figure 4

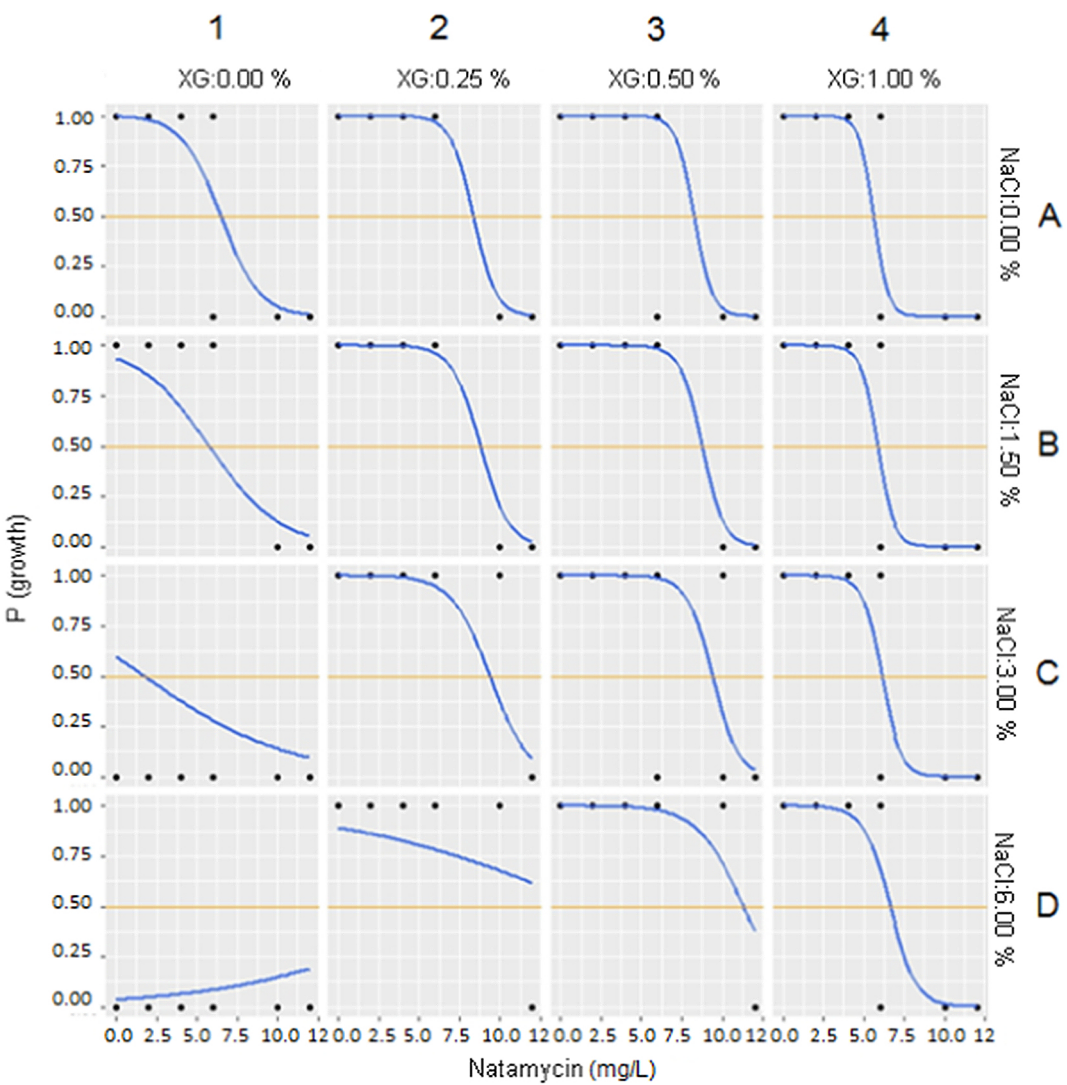
1
2
3
4
XG:0.00 %
XG:0.25 %
XG:0.50 %
XG:1.00 %
NaCl:0.00 %
NaCl:1.50 %
NaCl:3.00 %
NaCl:6.00 %
A
B
C
D
P (growth)
1.00
0.75
0.50
0.25
0.00
0.0 2.5 5.0 7.5 10.0 12
Natamycin (mg/L)

Figure 5