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Effects of water activity on the performance of potassium sorbate and natamycin as preservatives against cheese spoilage moulds

P. Marín[1], C . Ginés[2], P. Kochaki[2], M. Jurado[2†]

[1]Plant Response Biotech S.L., Centro de Biotecnología y Genómica de Plantas, Campus de Montegancedo, 28223 Pozuelo de Alarcón, Spain

[2]Department of Chemistry and Food Technology, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas, Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain

Abstract

This work investigated the effects of the food preservatives potassium sorbate and natamycin, combined with different levels of ionic (sodium chloride) and non-ioinic (glycerol) water activity (a_w), on growth of fungi involved in cheese spoilage. In general, the combined effect of water stress and presence of preservatives enhanced fungal inhibition. However, some doses of potassium sorbate (0.02%) and natamycin (1, 5 and 10 ppm) were able to stimulate growth of* Aspergillus varians, Mucor racemosus, Penicillium chrysogenum *and* P. roqueforti *at a_w values in the range of 0.93–0.97.* P. solitum *was the only species whose growth was consistently reduced by any doses of preservative. The results also showed that sodium chloride and glycerol differentially affected the efficacy of preservatives. This study indicates that a_w of cheese is a critical parameter to be considered in the formulation of preservative coatings used against fungal spoilage.

Keywords

antifungals • cheese • food spoilage • preservation • water activity

Introduction

Mould spoilage is one of the major problems causing deterioration of cheese. Development of fungal growth can occur virtually at any point of the ripening and storage stages and may cause undesirable effects such as off-odours and flavours, anomalous textures, discolourations and accumulation of mycotoxins (Sengun *et al*., 2008). Such defects are economically important since they are responsible for consumer rejection.

Different strategies can be implemented to improve the preservation of cheese and prevent the growth of moulds. A good cleaning and sanitising programme of the ripening rooms might help to reduce dispersible fungal spores present in the air and on the shelves (Ropars *et al*., 2012), although strict hygiene practices are difficult to maintain on a regular basis. In addition, cleaning and disinfecting might not be sufficient, considering that mould spores can also be transferred directly from milk to cheese (Lavoie *et al*., 2012; Panelli *et al*., 2014). Brushing of cheese wheels might help to remove mycelia from the rind but is a time-consuming activity, especially in the case of cheeses that are ripened for very long periods. Other practices, such as exclusion of air from the cheese through vacuum packaging, can also help to minimise mould growth. However, this procedure is normally done when cheeses have completed their ripening process, since vacuum packaging can slow down chemical and microbiological transformations necessary for the generation of volatile compounds (Andiç *et al*., 2011). In addition, vacuum packaging is not suitable for soft cheeses because it may induce undesirable changes in their textural properties (Pantaleão *et al*., 2007). The addition of food preservatives with fungistatic activity, though controversial, is still one of the best approaches for improving the keeping quality of cheese. Nevertheless, additives need to be used responsibly to ensure they comply with food regulations, especially those regarding the maximum usable dose. In most countries, the only preservatives authorised in cheese are natamycin and weak organic acids (sorbic or propionic acid) and their salts (sorbates and propionates) (Stark and Tan, 2003).

Besides preservatives, environmental factors might have a major impact on fungal growth. These factors include, among others, temperature, pH and availability of nutrients. Recently, it has been reported that water activity (a_w) is a key factor that modulates the growth of fungi associated with cheese (Marín *et al*., 2014). The status of water in cheese is extremely variable and depends not only on the variety of cheese considered but also on the stage of maturation (Gaucel *et al*., 2012; Pajonk *et al*., 2003; Saurel *et al*., 2004). During cheese ripening, a number of complex processes that involve chemical and biochemical

†Corresponding author: Miguel Jurado

E-mail: miguel.jurado@upm.es

reactions, mainly proteolysis and lipolysis, are responsible for the accumulation of low-molecular-weight compounds (Duggan *et al*., 2008). These events, together with water loss and diffusion of the sodium chloride (NaCl) added during manufacture, determine the occurrence of wide fluctuations in the concentration of ionic and non-ioinic osmolytes and, subsequently, on the levels of ionic and non-ionic a_w (Duggan *et al*., 2008) This variability influences the distribution of mycobiota involved in cheese spoilage because fungal species are differentially affected by ionic and non-ionic compounds (Marín *et al*., 2014).

The study of the interaction between fungistatic preservatives and environmental factors might be useful for predicting their efficacy, and thus there have been a number of previous studies on this subject (Guynot *et al*., 2005; Huang *et al*., 2009; Marín *et al*., 2002; Suhr and Nielsen, 2004). However, to our knowledge, none of them have focussed on fungi associated with cheese. Therefore, the objective of the present study was to evaluate the effect of different regimes of ionic and non-ionic a_w on the performance of potassium sorbate (PS) and natamycin as preservatives against mould species involved in cheese spoilage.

Materials and methods

Preparation of media

Sabouraud dextrose agar (Oxoid, Madrid, Spain) was used in this study. The value of a_w was modified with the ionic solute NaCl or the non-ionic solute glycerol to a_w values of 0.99, 0.97, 0.95 and 0.93. These solutes were not added to the control medium (a_w=0.996). The a_w of the media was checked with a hygrometer (AquaLab 3TE; Decagon Devices Inc., Pullman, Washington, USA).

PS (Sigma, Madrid, Spain) and natamycin of 50% purity (Danisco, Madrid, Spain) were aseptically added to the aforementioned media after autoclaving to give final concentrations of 0.02%, 0.1% and 0.2% (PS) or 1, 5 and 10 ppm (pure natamycin). No preservatives were added to the control media. The pH values of the media were adjusted to 5.4 with sterile 0.1 mol/L HCl after autoclaving, since the effectiveness of PS is known to be dependent on the pH (Plumridge *et al*., 2004).

Fungal isolates

Five fungal strains previously isolated from cheese (Marín *et al*., 2014) and comprising *Aspergillus varians* Mah1*, Mucor racemosus* Bet1*, Penicillium solitum* Mon2*, Penicillium roqueforti* Man1-3 and *Penicillium chrysogenum* Qpe1 were used for this study. Cultures were maintained on Sabouraud dextrose agar (Oxoid) at 4°C and stored as spore suspensions in 15% glycerol at –20 °C.

Inoculation, incubation and growth assessment

A 5-mm-diameter agar disc from the margin of a 7-d-old growing colony of each isolate grown at 20°C was used to centrally inoculate each replicate and treatment. The plates were incubated at 20°C for 10 d, and the experiment consisted of a fully replicated set of treatments with three replicates per treatment. Assessment of growth was made daily during the 10-d incubation period or until the colony reached the edge of the plate. Two diameters of the growing colonies were measured at right angles, and the radii of the colonies were plotted against time. Linear regressions were used to obtain growth rates from the slope of the line.

Statistical analysis of results

A two way analysis of variance (ANOVA) for each type of solute potential (a_w level × preservative dose) was performed separately for each species and fungistatic agent. A one-way ANOVA was performed when interaction of both factors (a_w × preservative dose) was significant. Subsequent *post hoc* analyses (Tukey's honest significance difference [HSD] tests of multiple comparisons) were carried out at a 95% confidence level. All sets of results were evaluated using Statgraphics Centurion XVII (Statistical Graphics Corp., Herndon, VA, USA) and SPSS 17.0.0 (Release 2008; SPSS Inc., Chicago, IL, USA).

Results

In general, and according to the two-way ANOVA performed separately for each fungal strain and preservative, the two factors ("a_w level" and "preservative dose") – as well as their interaction – had statistically significant effects on fungal growth of all the species tested (data not shown). Subsequently, a one-way ANOVA and their corresponding Tukey's HSD tests were carried out to determine which values of growth rate were different at a statistically significant level (Tables 1–5).

The contour maps of the relative growth rates in response to different a_w levels (0.996–0.93), a_w types (NaCl or glycerol) and different doses of PS (0%–0.2%) or natamycin (0–10 ppm) are shown in Figures 1–5. The standard deviations of the triplicates were typically <10% of the mean. When no preservatives were added, the decrease of a_w caused a different effect on fungal growth depending on the species tested. *M. racemosus* was the fastest-growing species but also the most affected by water stress, its growth being severely reduced at 0.95 and almost or completely inhibited at 0.93. Conversely, the reduction in a_w had a lower impact on growth of *P. chrysogenum* and no impact at all on growth of *P. solitum.*

The solute used to lower a_w also influenced the growth rates at a statistically significant level, with NaCl being more inhibitory

Table 1. Results of Tukey's HSD tests performed for *A. varians* growth rate (mm/d) and carried out separately for each factor (value of a_w and dose of fungistatic)*

***A. varians* Tukey's HSD tests**									
Potassium sorbate									
	Value of a_w (0%/0.02%/0.1%/0.2%)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	aaab	abcc	bac–	aaa–	aa–a	aabbc	acbde	abcb–	aa–a
Glycerol	aaab	aabb	abc–	aabc	abc–	babcc	bacde	aabcc	aa–a–
Natamycin									
	Value of a_w (0 ppm/1 ppm/5 ppm/10 ppm)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	abbb	abbb	baba	aaaa	cbaa	aabbc	abccc	abcbb	aaaaa
Glycerol	abbb	aabb	aaaa	babc	abcc	babcc	babbc	aaaab	aaabc

*Means that are not significantly different from each other are represented with the same letter. Significant differences ($P < 0.01$) among means are indicated by different letters, where the ranking of letters corresponds to the ranking of means from highest to lowest (a>b>c>d>e), and "–" indicates that no growth was detected.
HSD = honest significance difference.

Table 2. Results of Tukey's HSD tests performed for *M. racemosus* growth rate (mm/d) and carried out separately for each factor (value of a_w and dose of fungistatic)*

***M. racemosus* Tukey's HSD tests**									
Potassium sorbate									
	Value of a_w (0%/0.02%/0.1%/0.2%)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	aabb	abbc	aaab	abb–	——	aabc–	aabc–	aabc–	aaa––
Glycerol	aabb	aabb	aabb	bacd	aab–	bbacd	cbade	aaabc	abbc–
Natamycin									
	Value of a_w (0 ppm/1 ppm/5 ppm/10 ppm)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	abbc	abcd	abcc	abba	——	aabc–	aabc–	abcc–	abdc–
Glycerol	abbc	aabc	abcd	aaab	a––	bbacd	baab–	baab–	aaab–

*Means that are not significantly different from each other are represented with the same letter. Significant differences ($P < 0.01$) among means are indicated by different letters, where the ranking of letters corresponds to the ranking of means from highest to lowest (a>b>c>d>e), and "–" indicates that no growth was detected.
HSD = honest significance difference.

Table 3. Results of Tukey's HSD tests performed for *P. solitum* growth rate (mm/d) and carried out separately for each factor (value of a_w and dose of fungistatic)*

***P. solitum* Tukey's HSD tests**									
Potassium sorbate									
	Value of a_w (0%/0.02%/0.1%/0.2%)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	aaaa	aabb	abbb	abcd	aab–	aaaaa	baaaa	cdabc	bcac–
Glycerol	aaaa	aabb	abcd	aabb	abcc	aaaaa	cbaab	baaab	aaaaa
Natamycin									
	Value of a_w (0 ppm/1 ppm/5 ppm/10 ppm)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	aaaa	abbc	abcc	abcc	aaaa	aaaaa	bbaab	aabaa	aaaaa
Glycerol	aaaa	aabb	aabc	aabc	aab–	bbaab	ccaab	bbaab	aaaa–

*Means that are not significantly different from each other are represented with the same letter. Significant differences ($P < 0.01$) among means are indicated by different letters, where the ranking of letters corresponds to the ranking of means from highest to lowest (a>b>c>d>e), and "–" indicates that no growth was detected.
HSD = honest significance difference.

Table 4. Results of Tukey's HSD tests performed for *P. chrysogenum* growth rate (mm/d) and carried out separately for each factor (value of a_w and dose of fungistatic)*

***P. chrysogenum* Tukey's HSD tests**									
Potassium sorbate									
	Value of a_w (0%/0.02%/0.1%/0.2%)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	abcc	aabb	abbc	aabc	aa—	aaaab	baaab	bbab–	bbab–
Glycerol	abcc	aabb	abcc	babc	aaab	baacc	bbacc	bbaab	baaab
Natamycin									
	Value of a_w (0 ppm/1 ppm/5 ppm/10 ppm)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	aabc	aabb	abcc	aabb	aacb	aaaab	babbb	aaaab	bbbaa
Glycerol	aabc	aabc	abbc	babc	abcc	bbacc	aacbd	aaaab	bbbab

*Means that are not significantly different from each other are represented with the same letter. Significant differences ($P < 0.01$) among means are indicated by different letters, where the ranking of letters corresponds to the ranking of means from highest to lowest (a>b>c>d>e), and "–" indicates that no growth was detected.
HSD = honest significance difference.

Table 5. Results of Tukey's HSD tests performed for *P. roqueforti* growth rate (mm/d) and carried out separately for each factor (value of a_w and dose of fungistatic)*

***P. roqueforti* Tukey's HSD tests**									
Potassium sorbate									
	Value of a_w (0%/0.02%/0.1%/0.2%)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	aabc	aabb	aaaa	aaa–	aa –	aabcd	bacde	aabc–	aaa—
Glycerol	aabc	aaab	abcc	Babb	baaa	baacd	babac	babcd	baabc
Natamycin									
	Value of a_w (0 ppm/1 ppm/5 ppm/10 ppm)					**Dose of fungistatic (0.996/0.99/0.97/0.95/0.93)**			
	0.996	**0.99**	**0.97**	**0.95**	**0.93**	**0%**	**0.02%**	**0.1%**	**0.2%**
NaCl	abc–	ab—	ab—	a——	a——	aabcd	acb—	a——	———
Glycerol	abc–	abcc	abc–	abc–	ab—	baacd	aaabc	abbb–	–a——

*Means that are not significantly different one each other are represented with the same letter. Significant differences ($P < 0.01$) among means are indicated by different letters, where the ranking of letters corresponds to the ranking of means from highest to lowest (a>b>c>d>e), and "-" indicates that no growth was detected.
HSD = honest significance difference.

than glycerol, especially in the case of *M. racemosus* and *P. solitum*. On the contrary, *P. chrysogenum* was the most halotolerant species.

The "a_w level × preservative dose" interactions typically resulted in a higher inhibition of fungal growth. The growth rate decrease was, however, not always proportional to the reduction in a_w level nor to the increase in concentration of fungistatic. As a result, the shape of the growth rate curves were different in the media supplemented with NaCl or glycerol in comparison to control media (a_w=0.996) in the five species analysed.

Moreover, the use of certain doses of preservative at low a_w levels (in the range of 0.93–0.97) even resulted in a stimulation of fungal growth in *A. varians* (0.02% PS, 0.97 a_w NaCl; 1 and 10 ppm natamycin, 0.97 a_w NaCl; 1 ppm natamycin, 0.95 a_w, glycerol; 1, 5 and 10 ppm natamycin, 0.93 a_w, NaCl), *M. racemosus* (0.02% PS, 0.95 a_w, glycerol), *P. chrysogenum* (0.02% PS, 0.95 a_w glycerol; 1 ppm, 0.95 a_w NaCl) and *P. roqueforti* (0.02% PS, 0.95 a_w, glycerol; 0.02%, 0.1 and 0.2% PS, 0.93 a_w glycerol).

Discussion

Fungal spoilage of cheese occurs when moulds carried in milk or present in the chamber rooms are able to colonise the cheese rind, producing visible deterioration. Thus, spore germination and mycelial growth are subjected, on the one hand, to compositional intrinsic characteristics that depend on the cheese variety and, on the other hand, to the occurrence of extrinsic factors imposed from the outside. Study of the influence exerted by these factors is essential to

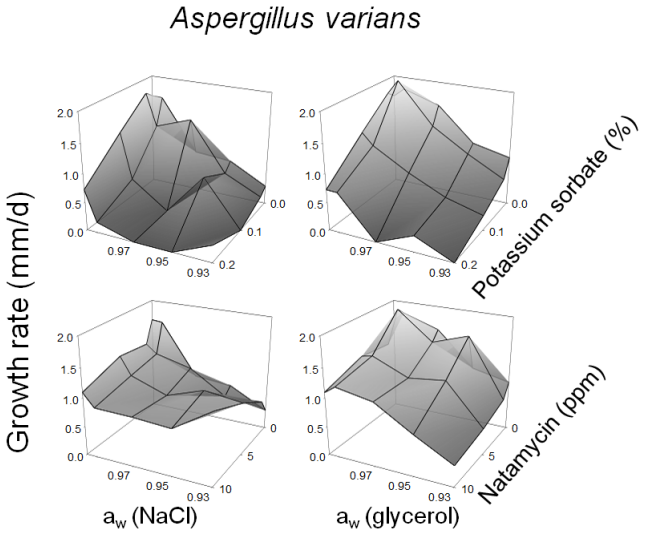

Figure 1. Contour maps of the effect of NaCl or glycerol on the growth rates of *Aspergillus varians* incubated for 10 d at different doses of the fungistatic agents potassium sorbate or natamycin and different a_w levels. The data shown are the mean of three replicates.

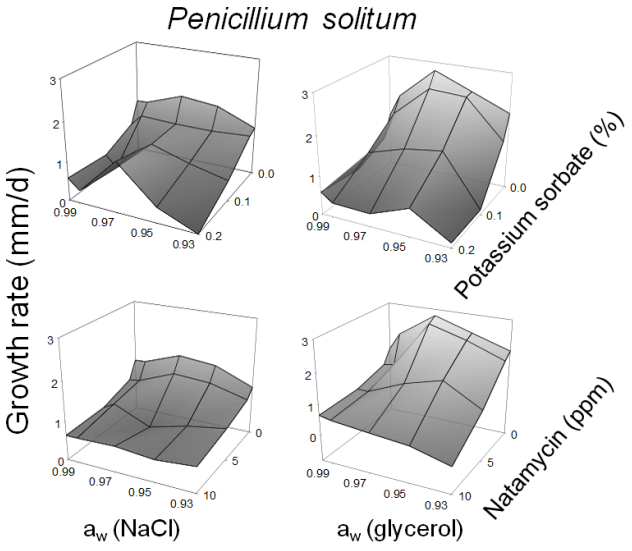

Figure 3. Contour maps of the effect of NaCl or glycerol on the growth rates of *Penicillium solitum* incubated for 10 d at different doses of the fungistatic agents potassium sorbate or natamycin and different a_w levels. The data shown are the mean of three replicates.

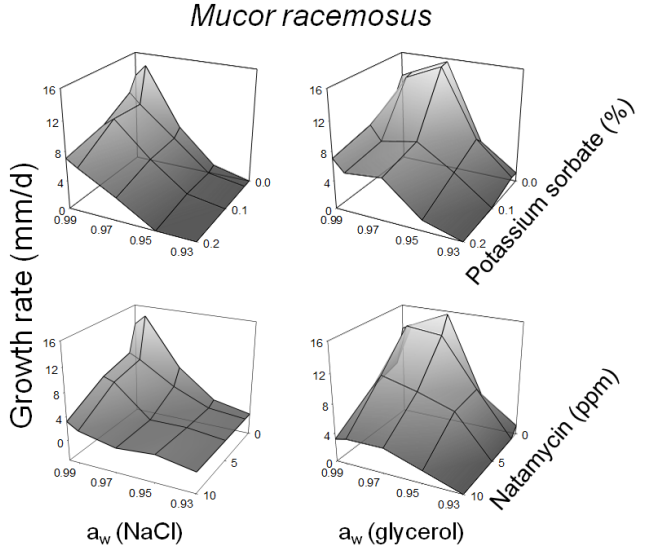

Figure 2. Contour maps of the effect of NaCl or glycerol on the growth rates of *Mucor racemosus* incubated for 10 d at different doses of the fungistatic agents potassium sorbate or natamycin and different a_w levels. The data shown are the mean of three replicates.

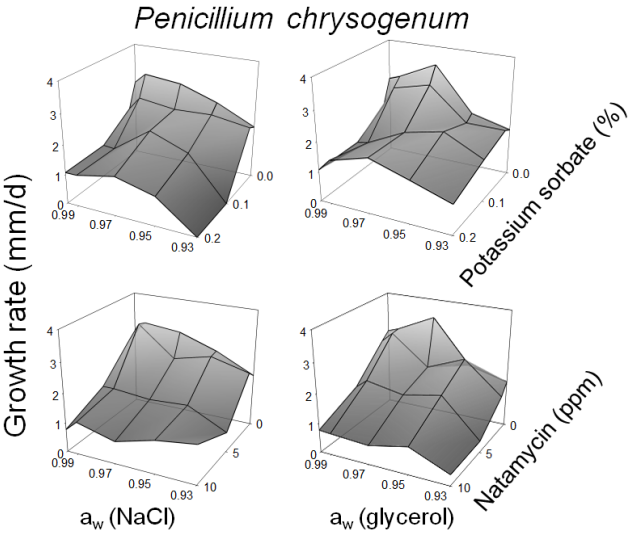

Figure 4. Contour maps of the effect of NaCl or glycerol on the growth rates of *Penicillium chrysogenum* incubated for 10 d at different doses of the fungistatic agents potassium sorbate or natamycin and different a_w levels. The data shown are the mean of three replicates.

predict the microbial stability of the product. According to the "Hurdle effect" concept (Leistner, 1992), many factors can be individually unable to completely inhibit microbial growth but the effectiveness can be enhanced when several factors occur simultaneously. In this study, we focussed on preservative concentration and a_w level because both factors might undergo quantitative fluctuations during cheese ripening and storage and thus might potentially influence the efficacy of these hurdles in preventing fungal spoilage. The concentrations at which preservatives are normally used in cheese are 0.02%–0.15% in the case of sorbates (Azza and Ahmed, 2010) and 1–20 ppm in the case of natamycin (Kallinteri *et al.*, 2013; Stark and Tan, 2003). The doses evaluated in this work might be therefore considered as ranging from standard to sub-

optimal. The study of the effect of low doses of preservatives is of interest not only because the modern trend is to reduce their concentration in foodstuffs due to consumer demands, but also because concentration of fungistatics can decrease due to diffusion phenomena within the bulk of cheese (Kristo *et al*., 2008). It should be noted that PS can be either incorporated in the whole cheese mass or impregnated in the rind, whereas natamycin is exclusively permitted for surface treatment (European Food Safety Agency [EFSA], 2009). In addition, the active concentration of PS can be reduced due to degradation of the molecule by fungal and bacterial activity (Mann and Beucheat, 2008; Montaño *et al*., 2013), whereas natamycin is broken down by UV light (Pedersen, 1992).

The results obtained in this work showed that, in general, both PS and natamycin were more efficient in inhibiting fungal growth when a_w was low. However, certain particular combinations of preservative doses and low a_w values resulted in the reduced efficacy of the preservative, and some combinations even stimulated fungal growth. Particularly, the lowest tested dose of PS, combined with certain low a_w values, was able to enhance fungal growth of *A. varians*, *M. racemosus*, *P. chrysogenum* and *P. roqueforti*. These results indicate that control of these moulds might be thus achievable through the addition of higher concentrations of the preservative. However, apart from the legal restrictions concerning the maximum permitted level of sorbates, the adverse effect of off-aromas and off-flavours that might result could make this approach impractical (Mann and Beucheat, 2008). Interestingly, a previous study that examined the interactions between PS and a_w on inhibition of fungi associated with bakery products also found a similar growth-stimulating effect of sub-optimal levels of PS at low a_w values (Marín *et al*., 2002). It is well known that some fungi are able to tolerate low concentrations of PS because they can degrade the molecule through decarboxylation and use it as a source of carbon (Montaño *et al*., 2013). It can be hypothesised that low a_w might selectively induce PS assimilation routes in some fungi, since there is increasing evidence that numerous metabolic pathways are differently expressed under different regimes of a_w (Bai *et al*., 2015; Zhang *et al*., 2014; Zhang *et al*., 2015). This field of research deserves further exploration.

At some values of reduced ionic and non ionic a_w, the species *A. varians*, *M. racemosus* and *P. chrysogenum* showed evidence of non-monotonic dose-growth response to natamycin. This kind of response is characterised by a curve whose slope changes direction within the range of tested doses, in this case – with low and high levels, causing less inhibition (or more stimulation) of fungal growth than intermediate levels. Non-monotonic curves are usually explained as the result of superimposition of monotonic dose responses of the component biological reactions (Conolly and Lutz, 2004).

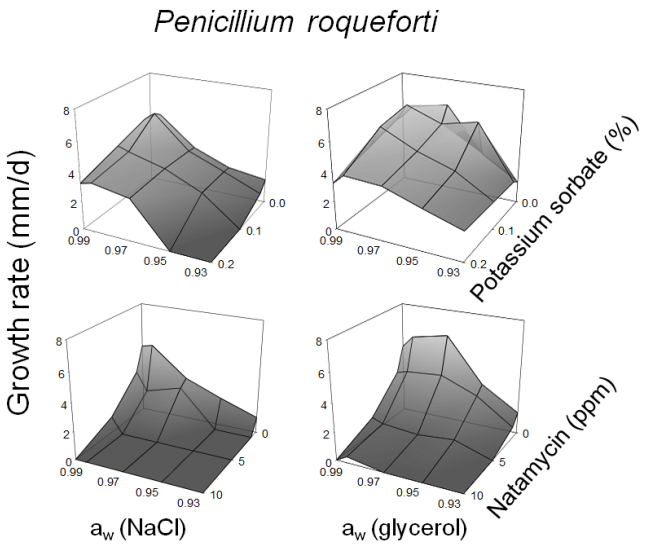

Figure 5. Contour maps of the effect of NaCl or glycerol on the growth rates of *Penicillium roqueforti* incubated for 10 d at different doses of the fungistatic agents potassium sorbate or natamycin and different a_w levels. The data shown are the mean of three replicates.

Whatever the precise causes, our results suggest that in order to effectively inhibit these species, it could be necessary to ensure a stable concentration of natamycin. Strategies such as encapsulation of the preservatives to achieve a sustained release of the molecule (Gortzi *et al*., 2006) and the use of polymeric substances able to prevent its migration (Fajardo *et al*., 2010) could be useful for this purpose.

The data obtained in this work suggest that, in the presence of sub-optimal or standard doses of preservative, the natural decrease of a_w that occurs during the ripening stage might trigger the growth of some fungal species if a certain threshold value is reached. It is possible that such a fact could be misinterpreted by cheese manufacturers as a decrease in the active concentration of the preservative, which could lead to unnecessary – and even counterproductive – re-application of preservative coatings. In our experience, re-application of preservative coatings is a very common practice in the cheese industry after an episode of re-emergence of mould contamination, especially in those varieties that are ripened for long periods of time. We think that both the formulation of preservative coatings and the timing of their application (and re-application) should be factors to carefully consider in any cheese industry.

Future research directed towards investigating the molecular mechanisms underlying resistance to food preservatives under water stress could also be beneficial. However, we must admit that this approach is overly complex, since the resistances detected varied according to the type of solute potential (ionic or non-ionic), as well as according to the species. Therefore, unfortunately, it seems very unlikely that guidelines in the

management of preservatives can be applicable to a wide range of cheeses. This highlights the necessity of gaining knowledge about the taxonomy of the spoiling mycobiota associated with a particular cheese variety. Nonetheless, a better understanding of the environmental factors modulating the dynamics of fungal populations in cheese would be determinant to achieving a higher level of performance of preservatives. The integration of this information could be extremely useful to develop *ad hoc* preservative formulations against fungal spoilage of different varieties of cheese.

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