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ORIGINAL ARTICLE

Effect of temperature, water activity, pH and some antimicrobials on the growth of *Penicillium olsonii* isolated from the surface of Spanish fermented meat sausage

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The effect of various factors and compounds used in the manufacturing of 'Cantimpalos chorizo', a fermented meat sausage made in Spain, on the growth of three strains of Penicillium olsonii *isolated from the surface of the sausage has been investigated. In addition, one strain of* Penicillium nalgiovense *(ATCC 66742), used as starter culture in fermented foods, has been included in the study. Fungi were grown under various ecological conditions (temperature, water activity and pH) and in the presence of the main antimicrobial compounds (sodium chloride, sodium nitrite and potassium nitrate) and spices (paprika, oregano and garlic) that are added to the initial sausage mixture. The effect of different combinations of some of the factors was also investigated.*

The results showed that at the levels found during the manufacturing of the sausage, the most influencing parameters on the growth of both species were water activity, temperature and sodium chloride. Both P. olsonii *and* Penicillium nalgiovense *were capable of growing at 10°C.* Penicillium nalgiovense *and one of the natural* P. olsonii *strains could also grow at the lowest water activity tested (0·86). However, the optimum water activity was higher for both species (0·92 for* P. olsonii *and close to 1 for* P. nalgiovense*). The pH, nitrates and nitrites, and the spices tested had no or a very little effect on the growth of both species. Differences among the naturally isolated strains were not significant (*$P > 0{\cdot}05$*) in most experiments. Also, in general terms, the behaviour of our isolates was very similar to the reference strain of* P. nalgiovense*, which indicates closeness to this species from a technological point of view and suggests the possibility of the use of* P. olsonii *as starter culture.*

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Introduction

In a previous study (López-Díaz et al. 2001), it was shown that *Penicillium olsonii* and *Penicillium commune* were the dominant *Penicillium* species on the surface of 'Cantimpalos chorizo', a Spanish fermented meat sausage which is characterized by its white surface.

The importance of genus *Penicillium* on the surface microbiota of several varieties of fermented meat sausages has been stated in several studies (Leistner and Eckardt 1979, Mutti et al. 1988, Leistner 1990), that have revealed

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the prevalence of the species *Penicillium nalgiovense* and *Penicillium chrysogenum* in the isolates.

The possible role of *Penicillium* spp. on the ripening process of fermented meat sausages, apart from the formation of the typical appearance, is multiple (i.e. antioxidative effect, formation of a favourable 'microclimate', development of a characteristic flavour due to the decomposition of proteins, fat and lactic acid; Philipp and Pedersen 1988). In addition, over the last decade, several authors (Geisen et al. 1992, Leistner 1990, Singh and Dincho 1994) have reported a protective effect of some of these moulds against some undesirable microorganisms.

The presence of penicillia on the surface of these varieties of meat sausage can be considered as being desired, although this mycoflora must be controlled in order to prevent the development of toxigenic and/or spoiling species and strains. It is generally accepted that the strains to be used in fermented foods must be non-toxigenic (incapable of producing mycotoxins) and non-toxic for consumers. In previous studies (López-Díaz et al. 2001), it was found that the *P. olsonii* isolates were non-producers of the most common mycotoxins produced by *Penicillium* subgenus *Penicillium*, and the extracts of our strains were non-toxic on *Artemia salina* larvae. In contrast, all *P. commune* strains isolated were cyclopiazonic acid producers. Initially, the use of these *P. olsonii* isolates in the manufacture of Cantimpalos chorizo could be considered but a previous research into the physiological behaviour of the strains isolated should be carried out.

Temperature, water activity and pH are among the critical parameters that affect mould growth in meat and meat products (Jay 1987). The main additives used during the manufacturing of Cantimpalos chorizo are sodium chloride, sodium nitrite, potassium nitrate, paprika, oregano and garlic (MAPA 1983). Although the physiological characterization of *P. nalgiovense* has been studied recently, using different combinations of factors (Haasum and Nielsen 1998a, 1998b), no data on the physiology of *P. olsonii* are known at present.

The results found on an ecological study of *P. olsonii* strains isolated from the surface of Cantimpalos chorizo are reported here, comparing them to one reference strain of *P. nalgiovense* (a species used as surface starter culture in the manufacture of salami, a similar variety of fermented meat sausage). Values of the parameters investigated are within the range that could be found in the Cantimpalos chorizo.

Materials and Methods

Strains

Three strains of *P. olsonii* isolated from the surface of Cantimpalos chorizo (López Díaz et al. 2001) were investigated (CH11A, CH18A and CH54A). In addition, a reference strain of *P. nalgiovense* (American Type Culture Collection, ATCC 66742), used as starter culture in fermented foods, was included in the study. Cultures were maintained at 5°C on slants of Malt Extract Agar (MEA, Oxoid, Basingstoke, Hampshire, England).

Effect of various parameters

The effect on the growth of the four *Penicillium* strains of the following factors was investigated: pH [4·5, 5·0, 5·5 (control) and 6·0], using NaOH 0·1 M and ClH 0·1 M to adjust pH; temperature [$10 \pm 0{\cdot}5$°C and $15 \pm 0{\cdot}5$°C, $25 \pm 0{\cdot}5$°C (control)] and water activity [a_w, 0·92, 0·90, 0·88, 0·86, 0·99 (control)], using an Aqua-Lab device (CX2, Decagon Devices Inc., Pullman, Washington, USA) to measure a_w of the prepared media. Glycerol (Acofarma, Madrid, Spain) was used to adjust the water activity of the medium.

Effect of different antimicrobial compounds

The growth of the four strains was assessed in the presence of the following compounds: NaCl (1·0, 2·0, 2·5 and 3·0%); a mixture of KCl and NaCl (50 : 50, w:w) (1·0, 2·0 and 3·0%); sodium nitrite (50, 75 and 100 ppm); potassium nitrate (50, 100 and 180 ppm); two combinations of paprika (dried and ground ripe fruit of *Capsicum annum*, from La Vera, Cáceres, Spain), oregano (dried and ground leaves of *Origanum* spp. imported from Chile) and garlic (dehydrated bulbs of *Allium sativum* imported from China): spices a (2·4%, 0·01% and 0·01%, respectively), and spices b (3%, 0·12%, 0·5%, respectively). Nitrate and nitrite solutions of a suitable concentration were added to the sterile medium after sterilization by filtration through a 0·22-μm pore size membrane filter; paprika, oregano and garlic were added to the sterile medium as water suspensions of a suitable concentration treated by tyndallization.

Finally, two combinations of salts and spices were tested at low (15°C) and high (25°C) temperatures. Mixture A: low concentrations of NaCl (1·0%), sodium nitrite (50 ppm), potassium nitrate (50 ppm), paprika (2·4%), oregano (0·01%) and garlic (0·01%). Mixture B: high concentrations of NaCl (3·0%), sodium nitrite (100 ppm), potassium nitrate (180 ppm), paprika (3·0%), oregano (0·12%) and garlic (0·5%).

In all the experiments, Malt Extract Agar (MEA, Oxoid) was used as the basal medium and as the control, using plates of 10 cm diameter, which were spot inoculated with a loop of a conidial suspension of each strain studied. The suspension was prepared from 7 day cultures on MEA slants, to which 5 ml of a sterile 0·01% Tween 80 solution was added to obtain an approx. 10^4 conidia/ml concentration. The diameter of colonies, in millimeters, was measured daily (in duplicate), from the 2nd to the 9th day or to the 18th whenever necessary. All experiments were performed in duplicate. From the growth curves obtained, the rate of growth (mm day^{-1}) was calculated by a regression analysis performed using the Statistica version 5.0, for Windows software (StatSoft, Tulsa, Oaklahoma, USA). Statistical differences in the rate of growth were calculated by a *t*-test (Statistica 5.0).

Results and Discussion

Figure 1 shows the effect of temperature on growth rate of the *Penicillium* strains. Similar growth patterns were observed for all strains, all of them being capable of growing at 10°C on MEA, although at a very low rate (around 1·2 mm day^{-1}). Growth rates increased significantly with temperature: 2–2·5 mm day^{-1} at 15°C and 3·6–4·0 mm day^{-1} at 25°C.

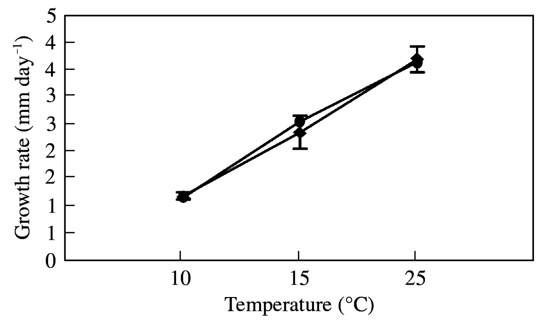

Figure 1. Effect of temperature on the growth rate of *Penicillium olsonii* isolated from the surface of Cantimpalos chorizo (mean ± standard deviation of three strains, two experiments each strain) and *Penicillium nalgiovense* (ATCC 66742, mean of two experiments). *Penicillium olsonii* (◆); *Penicillium nalgiovense* (●).

Most penicillia have their low minimal temperature in the refrigeration range, with some of them being capable of growing even below 0°C (Mislivec and Tuite 1970). Cantimpalos chorizo is a variety of meat sausage which is ripened at 14–18°C for about 20 days, after a short period (30 h) of drying at 25°C, to allow for lactic fermentation and a subsequent decrease of pH. According to our study, these ripening temperatures used would allow the growth of the *Penicillium* species found on the surface at half the rate obtained at optimum temperature.

The effect of water activity on the *Penicillium* strains investigated is shown in Fig. 2. Only two strains (CH54A and *P. nalgiovense*) were capable of growing in MEA with a water activity of 0·86, although at a very low rate (0·73 and 0·15 mm day^{-1}, respectively). Increasing the water activity resulted in an increase in the growth rate in both species. However, in the strains isolated from the sausage, the maximum rate (4·20 mm day^{-1}) was reached at 0·92, although differences from control (MEA, water activity close to 1) were not statistically significant ($P > 0·05$). On the other hand, the maximum rate of *P. nalgiovense* was found in the control (4·05 mm day^{-1}). Most *Penicillium* strains have an optimal a_w for growth of 0·97–0·98 (Hocking and Pitt 1979).

Conidial fungi are among the organisms capable of growing below 0·90 (Pitt and Hocking 1997), and most *Penicillium* spp. have a minimal

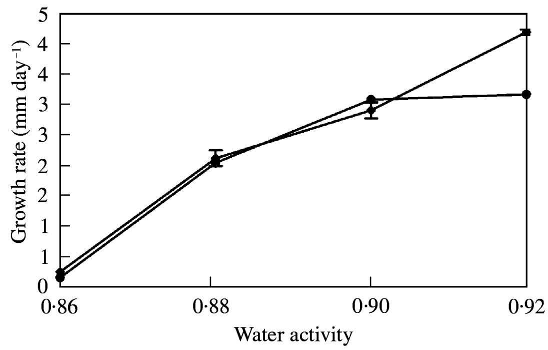

Figure 2. Effect of water activity on the growth rate of *Penicillium olsonii* isolated from the surface of Cantimpalos chorizo (mean ± standard deviation of three strains, two experiments each strain) and *Penicillium nalgiovense* (ATCC 66742, mean of two experiments). *Penicillium olsonii* (◆); *Penicillium nalgiovense* (●).

water activity for growth between 0·82 and 0·86 (Northolt et al. 1995). Our results suggest that at least some strains of *P. olsonii* and *P. nalgiovense* are capable of growing below 0·86. Cantimpalos chorizo is characterized by a fairly low water activity in the product at the time of consumption (below 0·90; Sanz et al. 1991). This low water activity could be one of the factors that would explain the prevalence of these *Penicillium* spp. on the surface of the sausage.

Table 1 shows the effect of pH on the growth of the strains investigated. Very little effect of the pH levels studied was found on the growth of the strains investigated. The highest average rate for *P. olsonii* was observed at pH 4·5; however, at this pH level, *P. nalgiovense* exhibited the minimum rate. One of the *P. olsonii* strains (CH54A) also grew more rapidly than the rest of *P. olsonii* strains and than *P. nalgiovense* at all pH levels studied (the growth rates were 4·41, 4·31, 4·38 and 4·82 mm day^{-1} at pH 4·5, 5·0, 5·5 and 6·0, respectively).

When the effect of pH in the presence of sodium chloride (3·0%) was investigated, no differences were found either in *P. olsonii* or in *P. nalgiovense* (Table 1). Various authors have also found a very low influence of pH on growth of several mould species (Buchanan and Ayres 1975, Holmquist et al. 1983, Wheeler et al. 1991), including *Penicillium* spp. (Thompson et al. 1993). The optimum growth rate of most fungi occurs at about pH 5·0 (Pitt and Hocking 1997) and, as we found in our study, both *P. nalgiovense* and *P. olsonii* could exhibit a good growth at the pH found in Cantimpalos chorizo (about 5·0; Sanz et al. 1991).

Table 1. Effect of pH and a combination of pH and sodium chloride on the growth rate of three strains of *Penicillium olsonii* isolated from the surface of Cantimpalos chorizo and one strain of *Penicillium nalgiovense*

pH	Growth rate (mm day^{-1})[a]	
	P. olsonii ($n = 3$)	*P. nalgiovense* ($n = 1$)[b]
C (5·5)[c]	$3·99 \pm 0·41^{B}$	$3·97 \pm 0·12^{B}$
4·5	$4·30 \pm 0·14^{A}$	$3·50 \pm 0·05^{B}$
5·0	$4·05 \pm 0·29^{B}$	$3·92 \pm 0·06^{AB}$
6·0	$4·21 \pm 0·71^{AB}$	$4·04 \pm 0·01^{A}$
C (5·5, 0% NaCl)[c]	$3·34 \pm 0·31^{C}$	$3·63 \pm 0·14^{C}$
4·5+NaCl[d]	$7·31 \pm 0·31^{A}$	$7·22 \pm 0·06^{A}$
5·0+NaCl[d]	$7·80 \pm 0.18^{B}$	$7·53 \pm 0·04^{B}$
6·0+NaCl[d]	$7·63 \pm 0·30^{AB}$	$7·73 \pm 0·01^{AB}$

[a]Data are presented as mean ± s.d. of growth rates (mm day^{-1}) (experiments in duplicate). For the same experiment, values in a column followed by the same capital letter do not differ significantly ($P > 0·05$); [b]*Penicillium nalgiovense* ATCC 66742; [c]C, controls; [d]NaCl 3·0%.

A stimulating effect of sodium chloride on the growth of the *Penicillium* strains was evident at all concentrations and in both species investigated (Fig. 3). Maximum growth rate was observed with salt concentrations ≥2·5%. When 1·0% salt was added to the basal medium (MEA), an increase of 40% on the rates of growth was observed (statistically significant, $P < 0·05$). However, another increase of 1·0% in NaCl caused a rise in the growth rate of *P. olsonii* strains of 13·7% ($P < 0·05$); similar figure for *P. nalgiovense* was 5·0% (statistically significant, $P < 0·05$). Subsequent addition of NaCl caused a very low increase in the growth rate (differences were not statistically significant, $P > 0·05$, among the rates found in presence of 2·0, 2·5 and 3·0% of salt). Finally, the replacement of 50% NaCl by KCl did not significantly affect ($P > 0·05$) the growth rate of all *Penicillium* strains at all the assayed concentrations (1·0, 2·0, 2·5 and 3·0%).

Sodium chloride is added to the initial sausage mixture of Cantimpalos chorizo at a concentration of 2·0% (MAPA 1983) and the final

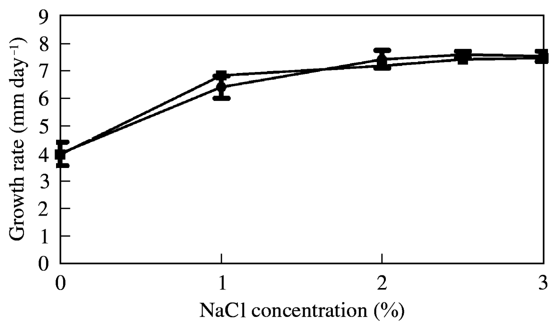

Figure 3. Effect of sodium chloride on the growth rate of *Penicillium olsonii* isolated from the surface of Cantimpalos chorizo (mean ± standard deviation of three strains, two experiments each strain) and *Penicillium nalgiovense* (ATCC 66742, mean of two experiments). *Penicillium olsonii* (◆); *Penicillium nalgiovense* (●).

Table 2. Effect of nitrites and nitrates on the growth rate of three strains of *Penicillium olsonii* isolated from the surface of Cantimpalos chorizo and one strain of *Penicillium nalgiovense*

Nitrite or nitrate concentration (ppm)	Growth rate (mm day^{-1})[a]	
	P. olsonii ($n=3$)	*P. nalgiovense* ($n=1$)[b]
C[c]	$3{\cdot}99 \pm 0{\cdot}34^A$	$3{\cdot}97 \pm 0{\cdot}12^A$
$NaNO_2$, 50	$3{\cdot}98 \pm 0{\cdot}33^A$	$4{\cdot}01 \pm 0{\cdot}01^A$
$NaNO_2$, 75	$4{\cdot}02 \pm 0{\cdot}28^A$	$4{\cdot}03 \pm 0{\cdot}06^A$
$NaNO_2$, 100	$4{\cdot}06 \pm 0{\cdot}30^A$	$4{\cdot}01 \pm 0{\cdot}11^A$
KNO_3, 50	$4{\cdot}01 \pm 0{\cdot}22^A$	$4{\cdot}02 \pm 0{\cdot}05^A$
KNO_3, 100	$3{\cdot}99 \pm 0{\cdot}34^A$	$4{\cdot}05 \pm 0{\cdot}06^A$
KNO_3, 180	$4{\cdot}00 \pm 0{\cdot}31^A$	$4{\cdot}17 \pm 0{\cdot}07^A$

[a]Mean ± s.d. (experiments in duplicate). Values in a column followed by the same capital letter do not differ significantly ($P > 0{\cdot}05$); [b]*Penicillium nalgiovense* ATCC 66742; [c]C, control.

content in the sausage was found to be 3·0% (Sanz et al. 1991). According to our results, the presence of this level of sodium chloride could encourage the growth of both species of *Penicillium*.

Other authors have found a similar stimulating effect of low salt concentrations on *Penicillium* growth. However, studies carried out on *Penicillium roqueforti* by Godinho and Fox (1981) and López-Díaz et al. (1996) showed a maximum growth rate at a lower NaCl concentration (1·0%) than those found by us for *P. olsonii* and *P. nalgiovense*.

Table 2 shows the effect of potassium nitrate and sodium nitrite on the growth of the four strains investigated. From the data found, it can be concluded that neither sodium nitrite nor potassium nitrate have a significant ($P > 0{\cdot}05$) effect even at the higher concentrations used (100 ppm and 180 ppm, respectively). The contents of these curing salts in Cantimpalos chorizo are between 50 and 200 ppm of potassium nitrate and 0·25 and 1·30 ppm of sodium nitrite (Sanz et al. 1991). Nitrates and nitrites are used in curing formulas of meat to stabilize red meat color, to inhibit spoilage and food pathogenic organisms and to contribute to flavour development (Jay 1992). Tompkin (1983) stated the lack of information on the effect of nitrite on yeasts and moulds. Since then, there have been no reports on this subject as far as the authors are aware of, but an inhibitory effect of potassium nitrate on aflatoxin production by *Aspergillus flavus* has been found (Strzelecki 1973).

As for the effect of the spices used in chorizo manufacturing, influence of both combinations used in our experiment on the *Penicillium* growth was very low (Table 3). In all cases, the addition of the spice mixtures reduced growth rate, but this reduction was low (less than 12%).

Spices are added to meat sausages as flavouring agents. Paprika, garlic and oregano are used in the manufacturing of chorizo. However, some spices have been shown to have antimicrobial activities, and therefore, some have been considered preservatives for many years. Oregano and garlic are the chorizo spices with a higher antimicrobial activity according to a study carried out by Davidson et al. (1983). More recently, several authors have found an inhibitory effect of several spices on mould growth. Kunz (1994) has found antimicrobial activity against some *Penicillium* species in bread, and Angulo and Gómez (1998) *in vitro*. Oregano caused a selective growth of *Penicillium citrinum* (Schmitz et al. 1993) and inhibited mycelial growth and sporulation of *Penicillium digitatum in vitro* (Tantaoui-Elaraki et al. 1993).

Finally, when a mixture of sodium chloride, sodium nitrite, potassium nitrate and the

Table 3. Effect of two mixtures of spices on the growth rate of three strains of *Penicillium olsonii* isolated from the surface of Cantimpalos chorizo and one strain of *Penicillium nalgiovense*

Mixtures	Growth rate (mm day^{-1})[a]	
	P. olsonii ($n=3$)	*P. nalgiovense* ($n=1$)[b]
C[c]	$3{\cdot}98 \pm 0{\cdot}29^{A}$	$3{\cdot}96 \pm 0{\cdot}12^{A}$
Spices a[d]	$3{\cdot}88 \pm 0{\cdot}20^{A}$	$3{\cdot}53 \pm 0{\cdot}15^{A}$
Spices b[e]	$3{\cdot}56 \pm 0{\cdot}12^{A}$	$3{\cdot}73 \pm 0{\cdot}06^{A}$

[a]Mean ± s.d. (experiments in duplicate). Values in a column followed by the same capital letter do not differ significantly ($P > 0{\cdot}05$); [b]*Penicillium nalgiovense* ATCC 66742; [c]C, control; [d]Spices a: paprika (2·4%), oregano (0·01%) and garlic (0·01%); [e]Spices b: paprika (3%), oregano (0·12%), garlic (0·5%).

three spices was tested, a stimulating effect on the growth of *Penicillium* was found both at 25°C and 15°C (Table 4). Growth at 25°C with mixture A was about 30% higher than in the basal medium (control) for both *Penicillium* species and the increase between mixture B and the control was even higher (around 40% for both species). At 15°C, differences with controls were lower, although statistically significant ($P < 0{\cdot}05$) for both *P. olsonii* and *P. nalgiovense* (for *P. olsonii,* 24% increase in relation to control for mixture A and 25·7% for mixture B; similar figures for *P. nalgiovense,* 13% for mixture A and 22% for mixture B). However, differences between mixtures A and B were not statistically significant ($P > 0{\cdot}05$; Table 4).

Taking into account the very low effect that nitrates, nitrites, paprika, oregano and garlic have on the growth of the *Penicillium* species investigated, the stimulating effect of both mixtures A and B could be mainly due to the effect of sodium chloride. However, significant differences ($P < 0{\cdot}05$) between the growth rates obtained for mixture A and NaCl 1% and between mixture B and NaCl 3%, at both 25°C and 15°C, were found, which suggests the effect of other factors apart from the sodium chloride content on the growth.

To sum up, our results show that at the levels that can be found in this variety of fermented meat sausage, temperature, water activity and sodium chloride are the most important parameters influencing the growth of *P. olsonii* strains isolated from the surface of Cantimpalos chorizo. These parameters also influence the growth of a *P. nalgiovense* strain used as starter culture in fermented foods.

We also found very little variations among the three naturally isolated strains of *P. olsonii* investigated and the behaviour of these isolates was also very close to the reference species used, *P. nalgiovense.* The fact that both species grow on very similar substrates and habitats could explain this resemblance.

Our isolates could be, therefore, very close to *P. nalgiovense*, from a physiological point of view, a fact that could encourage the use of some of these natural strains as surface starter cultures for Cantimpalos chorizo and similar varieties of fermented meat sausages.

Table 4. Effect of several factors on growth rate of three strains of *Penicillium olsonii* isolated from the surface of Cantimpalos chorizo and one strain of *Penicillium nalgiovense*

Temperature	Mixture	Growth rate (mm day^{-1})[a]	
		P. olsonii ($n=3$)	*P. nalgiovense* ($n=1$)[b]
25°C	C[c]	$3{\cdot}34 \pm 0{\cdot}31^{A}$	$3{\cdot}63 \pm 0{\cdot}14^{A}$
	Mixture A[d]	$4{\cdot}64 \pm 0{\cdot}25^{B}$	$5{\cdot}29 \pm 0{\cdot}28^{A}$
	Mixture B[e]	$6{\cdot}07 \pm 0{\cdot}42^{C}$	$6{\cdot}09 \pm 0{\cdot}16^{A}$
15°C	C[c]	$2{\cdot}34 \pm 0{\cdot}36^{A}$	$2{\cdot}55 \pm 0{\cdot}21^{A}$
	Mixture A	$3{\cdot}10 \pm 0{\cdot}49^{B}$	$2{\cdot}95 \pm 0{\cdot}10^{B}$
	Mixture B	$3{\cdot}15 \pm 0{\cdot}34^{B}$	$3{\cdot}27 \pm 0{\cdot}33^{AB}$

[a]Mean ± s.d. (experiments in duplicate). For each temperature, values in a column followed by the same capital letter do not differ significantly ($P > 0{\cdot}05$); [b]*Penicillium nalgiovense* ATCC 66742; [c]C, controls; [d]Mixture A: NaCl (1%), $NaNO_2$ (50 ppm), KNO_3 (50 ppm), paprika (2·4%), oregano (0·01%) and garlic (0·01%); [e]Mixture B: NaCl (3%), $NaNO_2$ (100 ppm), KNO_3 (180 ppm), paprika (3%), oregano (0·12%) and garlic (0·5%).

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