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## *Penicillium* species present in Uruguayan salami

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### ABSTRACT

The surface coverage of certain dry fermented sausages such as Italian salami by some species of *Penicillium* provides their characteristic flavor and other beneficial properties. One of them is the protective effect by means of a uniform film of white mold against undesirable microorganisms. The aim of this work was to identify and to isolate the fungal species present in mature Italian type of salami and to evaluate if it is possible to obtain some of them as starters. In addition, the effects of temperature (14 °C and 25 °C), water activity ( $a_w$ ) (0.90, 0.95 and 0.995) and 2.5 % sodium chloride (NaCl) on fungal growth were determined. Similarly, the proteolytic and lipolytic activity and the ability to produce toxic secondary metabolites were evaluated in order to characterize some possible starter strain. All species found belong to the genus *Penicillium*, including a performing starter as *Penicillium nalgiovense* and some potentially toxicogenic species. All the strains showed a higher growth rate at 25 °C. The production of extracellular proteases and lipases was significantly higher at 25 °C than at 14 °C with and without sodium chloride. Only *Penicillium expansum* produced patulin. On the other hand, *Penicillium griseofulvum* was the only species that produced cyclopiazonic acid but none of the strains produced penicillin. The species present on salami, *Penicillium nalgiovense*, *Penicillium minioluteum*, *Penicillium brevicompactum* and *Penicillium puberulum* were unable to produce any of the evaluated toxins. These findings suggest that some fungal isolates from the surface of salami such as *P. nalgiovense* are potentially useful as starters in sausage manufacture.

**Key words:** water activity, proteases, lipases, mycotoxins, fermented meat products

### RESUMEN

**Especies de *Penicillium* presentes en salamines uruguayos.** La cobertura de la superficie de los embutidos fermentados secos –como el salami tipo italiano– por algunas especies de *Penicillium* les proporciona un sabor característico y otras propiedades beneficiosas. Una de ellas es el efecto de protección contra microorganismos indeseables, al formarse una película blanca uniforme de mohos. El objetivo de este trabajo fue aislar e identificar los hongos filamentosos encontrados en la superficie de salamines tipo italiano y evaluar la posibilidad de obtener especies para utilizarse como cultivos iniciadores. Se determinó el efecto de la temperatura, la actividad de agua y del cloruro de sodio sobre el crecimiento fúngico. La actividad proteolítica y lipolítica y la capacidad de producir metabolitos secundarios tóxicos fueron evaluadas con el fin de caracterizar algunos posibles cultivos iniciadores. Todas las cepas fúngicas aisladas e identificadas correspondieron a especies del género *Penicillium*, algunas benéficas, como *Penicillium nalgiovense*, y otras potencialmente toxicogénicas. Estas cepas tuvieron diferentes tasas de crecimiento en respuesta a las diferentes condiciones de cultivo. Todas las cepas mostraron mayor crecimiento a 25 °C. La producción de proteasas y lipasas extracelulares fue significativamente mayor a 25 °C que a 14 °C. *Penicillium expansum* fue la única especie que produjo patulina y *Penicillium griseofulvum* fue la única que produjo ácido ciclopiazónico. Ninguna de las especies produjo penicilina. *Penicillium nalgiovense*, *Penicillium minioluteum*, *Penicillium brevicompactum* y *Penicillium puberulum* no produjeron ninguna de las toxinas evaluadas. Estos resultados sugieren que algunos aislamientos fúngicos, como *P. nalgiovense*, son potencialmente útiles como cultivos iniciadores en la fabricación de estos productos.

**Palabras clave:** actividad de agua, proteasas, lipasas, micotoxinas, productos cárnicos fermentados

### INTRODUCTION

Physical, chemical and biochemical changes during the manufacturing process of dry fermented meat products are responsible for obtaining an appropriately sound product (4). This process involves three stages: 1) mincing and mixing of ingredients, 2) fermentation and drying (26) and 3) microorganism colonization including filamentous fungi and yeasts, which is initiated after the stuffing (21). The water activity ( $a_w$ ) of salami that is initially around 0.98,

reaches 0.96-0.94 during the fermentation process, whereas the  $a_w$  is gradually reduced from 0.95 to 0.90 during the drying for the Italian type of salami produced in Uruguay.

The development of flavor in cured fermented meat products is a complex process in which lipolytic and proteolytic activities are the main pathways involved (6, 8, 29). Lipases from molds which grow on the surface of these meat products increase the levels of free fatty acids and, therefore, play a role in

determining the flavor and aroma of the product (34). Similarly, a relationship between the sensory properties of dry fermented meat sausages and the proteolytic activity of these molds was established. The presence of fungal mycelium on the surface of these products is also important, since it produces a protective effect against some undesirable microorganisms during the ripening process (19, 35). Mold coating on raw dry sausages is considered by manufacturers to be an indicator of good quality (35). Even though, there are many studies in the literature about the influence of the mold growth on the flavor of sausage (24, 25), there are very few studies on the proteolytic and lipolytic ability of fungi used as starters (25). On the other hand, the presence of filamentous fungi have several other positive effects such as antioxidant properties, water loss regulation, contribution to specific aroma of the final product (4, 20). In 2001, López-Díaz *et al.* (20), have pointed out that not only must fungal strains evidence a high rate of spore germination and mycelial growth, high proteolytic and lipolytic activities and uniform color of the mycelium to be used in the manufacturing product industry but they must also be atoxicogenic. An atoxygenic, proteolytic and lipolytic strain of *Penicillium aurantiogriseum* was tested by Bruna *et al.*, (7) for its ability to accelerate the production of volatile compounds and to improve the sensory properties of dry fermented sausages that were superficially inoculated with a spore suspension added with an intracellular cell free extract. Higher levels of lipid oxidation products were found in the aroma extracts of sausages without a mould cover. In contrast, branched aldehydes and alcohols presented higher concentrations in superficially inoculated and extract added sausages, while esters only showed higher concentration in the first ones. The sensory analysis showed that sausages prepared with both treatments received the highest scores in all the properties evaluated, which demonstrated both the potential of this mould as producer of volatile compounds and the effectiveness of combining both treatments.

The aim of this work was to identify and to isolate the filamentous fungi present in mature Italian type salami and to evaluate the possibility of obtaining species to be used as starter cultures. In addition, the effects of temperature (14 °C and 25 °C), water activity ( $a_w$ ) (0.90, 0.95 and 0.995) and 2.5 % sodium chloride (NaCl) on fungal growth were determined.

Similarly, the proteolytic and lipolytic activity and the ability to produce toxic secondary metabolites, such as patulin, ciclopiazonic acid and penicillin were evaluated in order to characterize some possible starter strain.

## MATERIALS AND METHODS

### Sampling and fungal isolation

Eighteen salami with spontaneous inoculation were obtained from two registered trademarks (A and B).

Fragments of 1 cm<sup>2</sup> from the center and both extremes were cut off from each salami surface, introduced in a solution of sterile distilled water with 0.1 % Tween 80 (Sigma-Aldrich Co, MO, USA) and vortexed during one minute. Dilutions were performed and 1ml of 10<sup>-3</sup> conidia was selected and staled on a Petri dish containing malt extract agar (MEA 2 %, Difco Laboratories, MI, USA), pH 4.5. Five replicates were performed. Colonies that emerged were transferred to a fresh medium (MEA 2 %).

The mould isolates belonging to the genus *Penicillium* were identified at species level by following Pitt's methodology (30, 31) which takes into account macroscopic, microscopic and eco-physiological characteristics. Spores of each isolate were inoculated on different culture media, different temperature and  $a_w$ .

One isolate of each species was used to evaluate the physiological characteristics.

### Effect of water activity, temperature and NaCl on fungal growth rate

The effect of  $a_w$  0.90, 0.95 and 0.995 (control), temperature (14 °C and 25 °C) and the presence of NaCl 2.5 % (J.T. Baker, México) in culture medium on the growth rate of *Penicillium* spp. were investigated. Ten  $\mu$ l of conidial suspension (10<sup>4</sup> conidia/ml) of each isolate was inoculated on MEA 2 % amended with an increasing volume of glycerol adjusting to different  $a_w$  according to the method described by Gervais *et al.* (13). To evaluate the effect of NaCl 2.5 % (the average concentration used in salami preparation) with an increasing volume of glycerol adjusting to different  $a_w$  according with Carrillo-Inungaray *et al.* (9). Plates were incubated at 14 °C and 25 °C and the diameter of colonies measured daily over the course of 7 days. Three replicates were performed for each experimental condition.

### Proteolytic and lipolytic activity

To determine the production of extracellular proteases casein-agar (2 %) (Merck, Darmstadt, Germany) was used (40) with and without NaCl, 2.5 %. Ten  $\mu$ l of a conidial suspension (10<sup>4</sup> conidia/ml) of each isolate was inoculated on culture medium and incubated at 14 °C and 25 °C for 7 days. The protease production was detected as clearer areas surrounding the colony, indicating that hydrolysis of the substrate had occurred (40).

Index of activity was calculated as:  $(d + h)/d$ , where d is the fungal colony diameter and h is the diameter of the clearance halo.

The lipolytic activity was determined using Tween 20 (1 %) (Sigma-Aldrich, MO, USA) as substrate with a basal medium containing 10 g peptone, 5 g NaCl, 0.1 g CaCl<sub>2</sub>·2H<sub>2</sub>O and 20 g of agar per liter of distilled water at pH 6. Ten  $\mu$ l of a conidial suspension (10<sup>4</sup> conidia/ml) of each isolate was inoculated on culture medium and incubated at 14 °C and 25 °C. The lipase enzyme production was detected by the formation of calcium salts crystals of lauric acid liberated by the enzyme activity around the colony due to complete fatty acid degradation (15).

Index of activity was calculated as:  $(d + h)/d$ , where d is the fungal colony diameter and h is the diameter of the halo formed by crystal precipitation.

Three replicates were performed for each experimental condition.

## Production of secondary metabolites

The toxin production of different strains was determined by evaluating the larvae mortality of brine shrimp larvae (*Artemia salina*) (14, 36). Flasks containing 25 ml yeast extract-sucrose (YES) (Difco Laboratories, MI, USA) were inoculated with a suspension of  $10^4$  conidia/ml of each different fungal species. Flasks were incubated in a shaker (New Brunswick Scientific, model 25KC, Edison, NT-USA) at 25 °C, 150 r.p.m. for 7 days. In order to remove the mycelium, 3 ml from each flask were centrifuged with a microcentrifuge (Heinle, model 7230 MR) at 10,000 r.p.m. for 10 minutes. The supernatant was filtered using 0.22  $\mu$ m Millipore filter (Millipore Corporation, MA, USA) and dilutions 1/10, 1/50 and 1/100 in sterile distilled water were performed.

Eggs of *A. salina* were placed in microplates with distilled water and NaCl 2.4 % and incubated during 24 hours at 25 °C. When eggs eclosion occurred, 20-30 larvae per well were placed in 2 ml of culture medium (YES) amended with 0.1 ml of each fungal species supernatant dilution. YES culture medium without inoculation as control was used. Six replicates were performed. The larvae were incubated at 25 °C. After 24 hours, larvae mortality was determined by observation under a stereo microscope (Nikon, model SMZ-U, Nikon Corp., Tokyo, Japan). The percentage of larvae mortality was calculated in relation to the control as follows:

% Mortality =  $100 - (\text{number of alive treated brine shrimp larvae} / \text{number of alive control brine shrimp larvae}) \times 100$ .

Ciclopiazonic acid (CPA) and patulin production from different isolates was determined by thin layer chromatography (TLC) (28). Isolates of fungal species were grown in YES and incubated at 25 °C and 14 °C for 10 days. CPA and patulin standards (Sigma-Aldrich, MO, USA) were prepared with chloroform 50  $\mu$ g/ml.

The CPA was revealed by spraying the plates with p-dimethylaminobenzaldehyde (1 %) (Sigma-Aldrich, MO, USA) in 75 ml of ethanol and 25 ml of hydrochloric acid. A blue band evidenced the presence of this mycotoxin (17). The evaluation of patulin production was revealed with an aqueous solution of methyl-2-benzothiazolinone hydrazone 0.5 % (MBTH) (Sigma-Aldrich, MO, USA) and finally the plates were dried at 130 °C for 15 minutes. Patulin was visualized as a yellow band (41).

To evaluate the ability to produce penicillin, isolates were grown on MEA for 7 days at both 25 °C and 14 °C temperatures. Disks with mycelium from the center of the colony were placed on nutrient agar (Difco Laboratories, MI, USA) previously inoculated with 0.1 ml of a 24-h culture of *Staphylococcus aureus* (ATCC 25923) sensible to penicillin and with 0.1 ml of a 24-h culture of *S. aureus* (ATCC 43300) resistant to the same antibiotic. Plates were incubated at 4 °C for 6 hours and then incubated at 37 °C for 24 hours (22). Three replicates were performed. Penicillin disks (Oxoid Ltd., CA, UK) with 6 mg of penicillin G were used as positive controls.

## Data Analysis

The relative density of each fungal strain was calculated as the number of isolates of each species  $\times$  100/total isolates.

The effect of temperature,  $a_w$  and NaCl on growth rate and the different ability to produce lipases or proteases by fungal isolates was evaluated using the non-parametric test of Kruskal-Wallis. All statistical tests were performed with the program Statistica 98 and  $p$  value  $< 0.05$  was considered as significant. To determine significant differences of the toxicogenic ability among fungal species an analysis of variance (ANOVA), previous transformation using the arcsen $\sqrt{x}$ , being  $x$  the survival percentages, was performed.

## RESULTS

### Fungal identification

Ten species of *Penicillium* were isolated from salami of two trademarks, A and B. There were differences in species compositions and number of isolates between salami of both trademarks.

The fungal species from salami produced by trademark A were *Penicillium aurantiogriseum*, *Penicillium expansum*, *Penicillium minioluteum* and *Penicillium nalgiovense*. *P. nalgiovense* was the most abundant species attaining 96 % of relative density.

From salami of trademark B, eight species of *Penicillium*: *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium corylophilum*, *Penicillium griseofulvum*, *P. minioluteum*, *P. nalgiovense*, *Penicillium pinophilum* and *Penicillium puberulum*, were found. *P. minioluteum* was the species (present) with the highest relative density (81 %).

### Effect of water activity, temperature and NaCl on fungal growth rate

All fungal species were able to grow at  $a_w$  0.995 and *P. minioluteum* isolates showed the highest growth rate at 25 °C. At 14 °C, all species have grown except *P. pinophilum* and *P. minioluteum* (isolate a).

Both isolates of *P. nalgiovense* showed the highest growth rate at  $a_w$  0.95. Conversely, a group of isolates (*P. aurantiogriseum*, *P. corylophilum*, *P. chrysogenum*, *P. griseofulvum*, *P. minioluteum* isolates a and b, *P. nalgiovense* isolates a and b, and *P. pinophilum*) did not grow at 14 °C and another group (*P. brevicompactum*, *P. expansum* and *P. puberulum*) showed some mycelial development.

No growth of any fungal isolate was observed at both temperatures.

The addition of NaCl 2.5 % did not stimulate growth of any fungal isolate at different temperatures ( $p > 0.05$ ) (Table 1).

### Proteolytic and lipolytic activity

*P. brevicompactum*, *P. nalgiovense* (isolate b), *P. pinophilum* and *P. griseofulvum* produced proteases at 25 °C, being the former the most active species. *P. brevicompactum* was the only species able to produce proteases at 14 °C (Table 2). The proteolytic activity with or without NaCl (2.5 %) at both temperatures was not significantly different ( $p > 0.05$ ) (Table 2).

At 25 °C, *P. aurantiogriseum*, *P. chrysogenum*, *P. expansum*, *P. griseofulvum*, *P. nalgiovense* (isolate a), *P. pinophilum* and *P. puberulum* showed the ability to produce extracellular lipolytic enzymes. At 14 °C

**Table 1.** Diameter of *Penicillium* colonies under different culture conditions after 7 days

Species	$a_w$ : 0.995		$a_w$ : 0.95		$a_w$ : 0.95 (with NaCl)	
	25 °C	14 °C	25 °C	14 °C	25 °C	14 °C
<i>P. aurantiogriseum</i>	8.62 ± 0.13 <sup>(1)(1)</sup>	0.67 ± 0.03 <sup>(1)(2)</sup>	7.33 ± 0.41 <sup>(1)(1)</sup>	0 <sup>(2)</sup>	7.10 ± 0.05 <sup>(1)(1)</sup>	0 <sup>(2)</sup>
<i>P. brevicompactum</i>	4.52 ± 0.12 <sup>(2)(1)</sup>	1.78 ± 0.15 <sup>(2)(2)</sup>	3.73 ± 0.23 <sup>(2)(1)</sup>	0.47 ± 0.16 <sup>(2)(2)</sup>	3.65 ± 0.30 <sup>(2)(1)</sup>	0.43±0.10 <sup>(2)(2)</sup>
<i>P. chrysogenum</i>	7.40 ± 0.10 <sup>(3)(1)</sup>	0.12± 0.03 <sup>(3)(2)</sup>	5.87 ± 0.15 <sup>(3)(1)</sup>	0 <sup>(2)</sup>	5.72 ± 0.28 <sup>(3)(1)</sup>	0 <sup>(2)</sup>
<i>P. corylophilum</i>	9.18 ± 0.42 <sup>(1)(1)</sup>	0.43 ± 0.06 <sup>(4)(2)</sup>	5.27 ± 0.12 <sup>(4)(1)</sup>	0 <sup>(2)</sup>	4.93 ± 0.08 <sup>(4)(1)</sup>	0 <sup>(2)</sup>
<i>P. expansum</i>	7.50 ± 0.20 <sup>(3)(1)</sup>	0.67 ± 0.03 <sup>(1)(2)</sup>	6.87 ± 0.12 <sup>(5)(1)</sup>	0.23 ± 0.16 <sup>(2)(2)</sup>	6.50 ± 0.10 <sup>(5)(1)</sup>	0.27±0.10 <sup>(2)(2)</sup>
<i>P. griseofulvum</i>	5.53 ± 0.08 <sup>(4)(1)</sup>	0.75± 0.00 <sup>(5)(2)</sup>	3.87 ± 0.25 <sup>(2)(1)</sup>	0 <sup>(2)</sup>	3.83 ± 0.38 <sup>(2)(1)</sup>	0 <sup>(2)</sup>
<i>P. minioluteum</i> (a)	11.87 ± 0.06 <sup>(5)(1)</sup>	0 <sup>(2)</sup>	2.45 ± 0.10 <sup>(6)(1)</sup>	0 <sup>(2)</sup>	2.35 ± 0.05 <sup>(6)(1)</sup>	0 <sup>(2)</sup>
<i>P. minioluteum</i> (b)	12.77 ± 0.18 <sup>(6)(1)</sup>	0.25± 0.00 <sup>(7)(2)</sup>	2.43± 0.28 <sup>(6)(1)</sup>	0 <sup>(2)</sup>	2.27 ± 0.08 <sup>(7)(1)</sup>	0 <sup>(2)</sup>
<i>P. nalgiovense</i> (a)	3.93 ± 0.19 <sup>(7)(1)</sup>	0.30±0.00 <sup>(6)(2)</sup>	5.28 ± 0.24 <sup>(4)(1)</sup>	0 <sup>(2)</sup>	5.47 ± 0.19 <sup>(3)(1)</sup>	0 <sup>(2)</sup>
<i>P. nalgiovense</i> (b)	5.13 ± 0.12 <sup>(8)(1)</sup>	0.28± 0.08 <sup>(9)(2)</sup>	7.08 ± 0.10 <sup>(1)(1)</sup>	0 <sup>(2)</sup>	7.25 ± 0.09 <sup>(8)(1)</sup>	0 <sup>(2)</sup>
<i>P. pinophilum</i>	8.87 ± 0.08 <sup>(9)(1)</sup>	0 <sup>(2)</sup>	1.23 ± 0.25 <sup>(7)(1)</sup>	0 <sup>(2)</sup>	1.12 ± 0.21 <sup>(9)(1)</sup>	0 <sup>(2)</sup>
<i>P. puberulum</i>	5.77 ± 0.12 <sup>(10)(1)</sup>	1.43± 0.38 <sup>(2)(2)</sup>	5.67± 0.42 <sup>(3)(1)</sup>	0.32 ± 0.16 <sup>(2)(2)</sup>	5.40 ± 0.26 <sup>(3)(1)</sup>	0.28±0.06 <sup>(2)(2)</sup>

Significative differences between columns are indicated by Arabic numbers in bold; significative differences within columns are indicated by Arabic numbers (non parametric test Kruskal-Wallis,  $p < 0.05$ ); (a), strain a; (b), strain b.

**Table 2.** Production of lipases and proteases at 25 °C and 14 °C with and without NaCl 2.5 %

Species	Lipases 25 °C		Proteases 25 °C		Proteases 14 °C	
	Without NaCl	With NaCl	Without NaCl	With NaCl	Without NaCl	With NaCl
<i>P. aurantiogriseum</i>	1.26 <sup>(1)</sup>	1.23 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>
<i>P. brevicompactum</i>	1 <sup>(2)</sup>	1 <sup>(2)</sup>	1.25 <sup>(2)(1)</sup>	1.24 <sup>(2)(1)</sup>	1.11 <sup>(2)(2)</sup>	1.10 <sup>(2)(2)</sup>
<i>P. chrysogenum</i>	1.32 <sup>(1)</sup>	1.32 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>
<i>P. corylophilum</i>	1 <sup>(2)</sup>	1 <sup>(2)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>
<i>P. expansum</i>	1.41 <sup>(3)</sup>	1.38 <sup>(3)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>
<i>P. griseofulvum</i>	1.27 <sup>(1)</sup>	1.28 <sup>(1)</sup>	1.04 <sup>(3)(1)</sup>	1.05 <sup>(3)(1)</sup>	1 <sup>(1)(2)</sup>	1 <sup>(1)(2)</sup>
<i>P. minioluteum</i> strain a	1 <sup>(2)</sup>	1 <sup>(2)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>
<i>P. minioluteum</i> strain b	1 <sup>(2)</sup>	1 <sup>(2)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>
<i>P. nalgiovense</i> strain a	1.23 <sup>(1)</sup>	1.20 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>
<i>P. nalgiovense</i> strain b	1 <sup>(2)</sup>	1 <sup>(2)</sup>	1.08 <sup>(4)(1)</sup>	1.07 <sup>(4)(1)</sup>	1 <sup>(1)(2)</sup>	1 <sup>(1)(2)</sup>
<i>P. pinophilum</i>	1.22 <sup>(1)</sup>	1.20 <sup>(1)</sup>	1.05 <sup>(3)(1)</sup>	1.05 <sup>(3)(1)</sup>	0 <sup>(2)</sup>	0 <sup>(2)</sup>
<i>P. puberulum</i>	1.63 <sup>(4)</sup>	1.59 <sup>(4)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>	1 <sup>(1)</sup>

Index: (diameter of growth + diameter of halo)/ diameter of growth. Significative differences between columns are indicated by Arabic numbers in bold; significative differences within columns are indicated by Arabic numbers (non parametric test Kruskal-Wallis,  $p < 0.05$ ).

no species was able to produce extracellular lipases (Table 2). Lypolytic enzyme production with and without NaCl and 25 °C was not significantly different ( $p > 0.05$ ).

**Production of secondary metabolites**

The test of toxicity showed that filtrates of *P. brevicompactum*, *P. minioluteum* (isolate b), *P. nalgiovense* (isolates a and b), and *P. puberulum* were not toxic to *A. salina* larvae. In contrast, filtrates from isolates of *P. aurantiogriseum*, *P. expansum*, *P. griseofulvum* and *P. pinophilum* were slightly toxic, whereas those of *P.*

*chrysogenum*, *P. minioluteum* (isolate a) and *P. corylophilum* were toxic (Table 3).

*P. expansum* was the only species that produced patulin at  $a_w$  0.995 at both temperatures. *P. griseofulvum* was the only species that produced CPA at 25 °C and  $a_w$  0.995. None of the fungal isolates were able to produce penicillin.

**DISCUSSION**

These results are in agreement with those obtained in studies conducted by other authors (2, 10, 37)

**Table 3.** Toxicity of culture filtrates of fungal isolates to brine shrimp larvae

Species	Mortality (%)	Toxicity
<i>P. nalgiovense</i> strain b	0.00	non toxic
<i>P. minioluteum</i> strain b	0.51	non-toxic
<i>P. nalgiovense</i> strain a	1.44	non-toxic
<i>P. brevicompactum</i>	2.21	non-toxic
<i>P. puberulum</i>	6.90	non-toxic
<i>P. griseofulvum</i>	18.64	slightly toxic
<i>P. aurantiogriseum</i>	19.26	slightly toxic
<i>P. pinophilum</i>	23.57	slightly toxic
<i>P. expansum</i>	33.73	slightly toxic
<i>P. chrysogenum</i>	61.95	highly toxic
<i>P. minioluteum</i> strain a	71.48	highly toxic
<i>P. corylophilum</i>	74.67	highly toxic

% of mortality =  $100 - (n.^{\circ} \text{ of alive treated brine shrimp larvae} / n.^{\circ} \text{ of alive control brine shrimp larvae}) \times 100$ ; 0-9 %: non-toxic, 10-49 %: slightly toxic, 50-89 %: toxic and 90-100 %: highly toxic (16).

where the dominant species belonged to the genus *Penicillium*, although the species were not always the same as those found in this work.

The high relative abundance of *P. nalgiovense* and *P. minioluteum* in salami of both trademarks indicates that these species can colonize the surface at high rate as was demonstrated by Castro *et al.* (11), who found that *P. nalgiovense* strains can also prevent contamination by other filamentous fungi in the ripening process of salami. Isolates of these species could be used as starters for salami preparation. Moreover, both *P. nalgiovense* isolates (isolates a and b) were not toxic to eukaryotic organisms although Larsen *et al.* (18) found that isolates of this species were toxic to cell cultures.

The reduction or absence of mycelial growth at 14 °C, as water activities decrease, is consistent with those obtained by López-Díaz *et al.*, Pose *et al.* and Santamarina *et al.* (21, 32, 33).

This could explain why as the maturation process advances, mycelial growth becomes limited. Ludeman *et al.* (23) described a stimulating effect of NaCl on growth of *Penicillium* species; however, in this work, NaCl did not show this effect. It is probable that the absence of proteolytic activity by *P. brevicompactum* at 14 °C is due to intra specific differences frequently found in the ability to produce proteases. Only *P. nalgiovense* isolates (isolate a) showed lipolytic activity consistent with Pose *et al.*'s findings (32).

Molds produce enzymes for the degradation of lipid and protein-matter, but studies show that proteolytic and lipolytic capabilities differ significantly between

strains and is highly dependent on media, pH and temperature (38).

Sodium chloride neither stimulated the proteolytic nor the lipolytic activity of *Penicillium* spp. incubated at 14 °C nor 25 °C. These results differ from those presented by Ludeman *et al.* (23), who found a stimulating effect of NaCl on the proteolytic and lipolytic activity. Some *P. chrysogenum* were found that could contribute to the development of desired texture and flavours in dry-cured meat products. The effect of *P. chrysogenum* on pork myofibrillar proteins has been assayed in a culture medium containing 5 % (w/v) NaCl and revealed to be responsible for hydrolysis of the main myofibrillar proteins except  $\alpha$ -actinin. Proteolysis led to increases in free amino acids (5).

*P. nalgiovense* was found to be positive to penicillin production in an agar assay and further examination for antibiotic production in a liquid culture with complex media designed for penicillin production, confirmed their ability for penicillin biosynthesis (1, 27). The absence of penicillin production by the species found here is an interesting and essential condition for using these isolates as starters but it is not possible to assure that they do not possess the genes for penicillin synthesis. For this reason it would be necessary to verify the presence of genes responsible for penicillin production by polymerase chain reaction (PCR) with specific primers to the  $\delta$ -(L- $\alpha$ -aminoadipyl)-L-cysteinyl-D-valine) synthetase gene (*pcbAB*), the isopenicillin-N-synthase gene (*pcbC*) and the acyl coenzyme A:6-aminopenicillanic acid acyltransferase gene (*pen DE*) from *P. chrysogenum* (12, 39). This condition represents a health risk for consumers (1, 3).

Low to high toxicity on *A. salina* larvae was evidenced by several of the *Penicillium* species tested here. The toxicity of *P. expansum* could probably be related to the ability of patulin production and that of *P. griseofulvum* to the ability to produce CPA and other mycotoxins, such as roquefortin and griseofulvin, which were not evaluated here (32).

Since the variability in mycobiota composition can affect the uniformity of product quality, it is very important that fungal strains be able to produce enzymes and to grow under different colonization conditions but it is yet more important that they be unable to produce toxins along the process of preparation of these meat products. In accordance with these exigencies, *P. nalgiovense* strains a and b could be selected as starters in salami production under natural conditions in Uruguay since they also have the ability to produce a good coverage.

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## REFERENCES

- Andersen SJ, Frisvad JC. Penicillin production by *Penicillium nalgiovense*. Lett Appl Microbiol 1994; 9: 486-8.
- Asefa DT, Gjerde RO, Sidhu MS, Langsrud S, Kure CF, Nesbakken T, Skaar I. Moulds contaminants on Norwegian dry-cured meat products. Int J Food Microbiol 2009; 128: 435-9.
- Asefa DT, Kure CF, Gjerde RO, Omer MK, Langsrud S, Nesbakken T, Skaar I. Fungal growth pattern, sources and factors of mould contamination in a dry-cured meat production facility. Int J Food Microbiol 2010; 140: 131-5.
- Bengoá MS. El salame y la tecnología aplicada en la elaboración de crudos madurados. Revista Prinal 2002; 20-3.
- Benito MJ, Córdoba JJ, Alonso M, Asensio MA, Núñez F. Hydrolytic activity of *Penicillium chrysogenum* on pork myofibrillar proteins. Int J Food Microbiol 2003; 89: 155-61.
- Bolmar García JT. Caracterización del sistema proteolítico de *Debaryomyces hansenii* aislada de embutidos curados. Tesis de Doctorado 2004. Universitat de Valencia. Servei de Publicacions.
- Bruna JM, Hierro EM, De La Hoz L, Mottram DS, Fernández M, Ordóñez JA. The contribution of *Penicillium aurantio-griseum* to the volatile composition and sensory quality of dry fermented sausages. Meat Sci 2001; 59: 97-107.
- Carrascosa Santiago AV. Cultivos iniciadores para la industria cárnica. En: Bejarano M, editores. Enciclopedia de la carne y de los productos cárnicos. Plasencia, España, Martín y Macías, Ediciones, 2001, p. 943-65.
- Carrillo-Inungaray ML, Ramírez-Zapata MR, Martínez-Castilleja JC. Efecto de solutos sobre el crecimiento de hongos deteriorativos de alimentos. Ciencia y Tecnología Alimentaria 2006; 5: 142-6.
- Castellari C, Quadrelli AM, Laich F. Surface mycobiota on Argentinean dry fermented sausages. Int J Food Microbiol 2010; 142: 149-55.
- Castro LC, Luchese RH, Martins JFP. Efeito do uso da cepa starter de *Penicillium nalgiovense* na qualidade de salames. Ciencia e Tecnologia de Alimentos 2000; 20: 40-6.
- Farber P, Geisen R. Antagonistic activity of the food-related filamentous fungus *Penicillium nalgiovense* by the production of penicillin. Appl Environ Microbiol 1994; 60: 3401-4.
- Gervais P, Molin P, Grajek W, Bensoussan M. Influence of water activity of a solid substrate on the growth rate and sporogenesis of filamentous fungi. Biotechnol Bioeng 1988; 31: 457-63.
- González AM, Presa M, Latorre ML, Lurá MC. Detección de metabolitos fúngicos con actividad tóxica mediante bioensayo sobre *Artemia salina*. Rev Iberoam Micol 2007; 24: 59-61.
- Hankin L, Anagnostakis SL. The use of solid media for detection of enzyme production by fungi. Mycologia 1975; 67: 597-607.
- Harwig J, Scott PM. Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. Appl Microbiol 1971; 21: 1011-6.
- Horn BW, Dorner JW. Regional differences in production of aflatoxin B<sub>1</sub> and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Appl Environ Microbiol 1999; 65: 1444-9.
- Larsen TO, Gareis M, Frisvad JC. Cell cytotoxicity and mycotoxin and secondary metabolite production by common *Penicillia* on cheese agar. J Agric Food Chem 2002; 50: 6148-52.
- Leistner L. Mold fermented foods: Recent developments. Food Biotechnol 1990; 4: 433-41.
- López-Díaz TM, González C, Rodríguez R, Encinas JP, Otero A. Utilización de mohos y levaduras en la elaboración de embutidos cárnicos fermentados. Alimentaria 2001; 1: 25-32.
- López-Díaz TM, González CJ, Moreno B, Otero A. Effect of temperature, water activity, pH and some antimicrobials on the growth of *Penicillium olsonii* isolated from the surface of Spanish fermented meat sausage. Food Microbiol 2002; 19: 1-7.
- López-Díaz TM, Santos JA, García-López ML, Otero A. Surface mycoflora of a Spanish fermented meat sausage and toxigenicity of *Penicillium* isolates. Int J Food Microbiol 2001; 68: 69-74.
- Ludeman V, Pose G, Pollio ML, Segura J. Determination of growth characteristics and lipolytic and proteolytic activities of *Penicillium* strains isolated from Argentinean salami. Int J Food Microbiol 2004; 96: 13-8.
- Ockerman HW, Céspedes Sánchez FJ, Ortega Mariscal MA, Leon Crespo F. The lipolytic activity of some indigenous Spanish moulds isolated from meat products. J Muscle Foods 2001b; 12: 275-84.
- Ockerman HW, Céspedes Sánchez FJ, Ortega Mariscal MA, Martín Serrano AM, Torres Muñoz MC, Leon Crespo F. Influence of temperature on proteolytic activity of indigenous Spanish molds in meat products. J Muscle Foods 2001a; 12: 263-73.
- Ordóñez Pereda JA, De La Hoz Perales L. Embutidos crudos curados. Tipos. Fenómenos madurativos. Alteraciones. En: Bejarano M, editores. Enciclopedia de la carne y de los

- productos cárnicos. Plasencia, España, Martín y Macías, Ediciones, 2001, p. 1063-90.
27. Papagianni M, Ambrosiadis I, Filiouis G. Mould growth on traditional Greek sausages and penicillin production by *Penicillium* isolates. *Meat Sci* 2007; 76: 653-7.
  28. Paterson RRM, Bridge PD. Biochemical techniques for filamentous fungi. IMI Technical Handbooks N.º 1. Surrey, UK, CAB International, 1994, p. 125.
  29. Philipp S, Pedersen PD. Mould cultures for the food industry. A short review with special reference to the cheese and sausage production. *Danish Dairy and Food Industry-Worldwide* 1988; 6: 10-2.
  30. Pitt JI. The genus *Penicillium* and its teleomorphic status *Eupenicillium* and *Talaromyces*, London, UK, Academic Press, 1979, p. 634.
  31. Pitt JI. A Laboratory Guide to Common *Penicillium* Species. CSIRO Division of Food Processing, North Ryde, NSW, Australia, 1988, p. 187.
  32. Pose G, Ludeman V, Pollio ML, Segura J. Micoflora autóctona de la superficie de embutidos secos fermentados. *La Industria Cárnica Latinoamericana* 2003; 129: 28-32.
  33. Santamarina Siurana MP, Roselló Caselles J, Barceló Cerdá S, Sillué SM. Efecto de la actividad de agua y de la temperatura sobre el comportamiento de *Penicillium oxalicum* frente a *Fusarium oxysporum*. *Rev Iberoam Micol* 2003; 20: 154-9.
  34. Selgas MD, Casas C, Toledo VM, García ML. Effect of selected mould strains on lipolysis in dry fermented sausages. *Eur Food Res Technol* 1999; 209: 360-5.
  35. Singh BJ, Dincho D. Molds as protective cultures for raw dry sausages. *J Food Prot* 1994; 57: 928-30.
  36. Skrobek A, Boss D, Défago G, Butt TM, Maurhofer M. Evaluation of different biological test systems to assess the toxicity of metabolites from fungal biocontrol agents. *Toxicol Lett* 2006; 161: 43-53.
  37. Sorensen LM, Jacobsen T, Nielsen PV, Frisvad JC, Koch, AG. Mycobiota in the processing areas of two different meat products. *Int J Food Microbiol* 2008; 124: 58-64.
  38. Sunesen LO, Stahnke LH. Mould starter cultures for dry sausages-selection, application and effects. *Meat Sci* 2003; 65: 935-48.
  39. Van den Berg MA. Functional characterisation of penicillin production strains. *Fungal Biol Rev* 2010; 24: 73-8.
  40. Varela, H. Producción de enzimas depilantes de pieles. Tesis para optar por el título de Doctor en Química. 1998. Facultad de Química. Universidad de la República Oriental del Uruguay.
  41. Vero S, Vázquez A, Cerdeiras MP, Soubes M. A rapid TLC-scanning method for the determination of patulin in apple products. *J Planar Chromatogr* 1999; 12: 172-174.