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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 ºC and 25 ºC), 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 ºC than at 14 ºC with and without sodium chloride. Only *Penicillium expansum* produced patulin. On the other hand, *Penicillium griseofulvum* was the only species that produced ciclopiazonic 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 salamín 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 ºC. La producción de proteasas y lipasas extracelulares fue significativamente mayor a 25 ºC que a 14 ºC. *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 ºC and 25 ºC), 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^2 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^{-3} 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 ecophysiological 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 ºC and 25 ºC) and the presence of NaCl 2.5 % (J.T. Baker, México) in culture medium on the growth rate of *Penicillium* spp. were investigated. Ten µl of conidial suspension (10^4 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 ºC and 25 ºC 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 µl of a conidial suspension (10^4 conidia/ml) of each isolate was inoculated on culture medium and incubated at 14 ºC 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 $CaCl_2.2H_2O$ and 20 g of agar per liter of distilled water at pH 6. Ten µl of a conidial suspension (10^4 conidia/ml) of each isolate was inoculated on culture medium and incubated at 14 º C and 25 º 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 µm 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 - (number of alive treated brine shrimp larvae/ number of alive control brine shrimp larvae) x 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 µg/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 a 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 x 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
P. aurantiogriseum	8.62 ± 0.13$^{(1)\,\mathbf{(1)}}$	0.67 ± 0.03$^{(1)\,\mathbf{(2)}}$	7.33 ± 0.41$^{(1)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	7.10 ± 0.05$^{(1)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$
P. brevicompactum	4.52 ± 0.12$^{(2)\,\mathbf{(1)}}$	1.78 ± 0.15$^{(2)\,\mathbf{(2)}}$	3.73 ± 0.23$^{(2)\,\mathbf{(1)}}$	0.47 ± 0.16$^{(2)\,\mathbf{(2)}}$	3.65 ± 0.30$^{(2)\,\mathbf{(1)}}$	0.43±0.10$^{(2)\,\mathbf{(2)}}$
P. chrysogenum	7.40 ± 0.10$^{(3)\,\mathbf{(1)}}$	0.12± 0.03$^{(3)\,\mathbf{(2)}}$	5.87 ± 0.15$^{(3)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	5.72 ± 0.28$^{(3)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$
P. corylophilum	9.18 ± 0.42$^{(1)\,\mathbf{(1)}}$	0.43 ± 0.06$^{(4)\,\mathbf{(2)}}$	5.27 ± 0.12$^{(4)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	4.93 ± 0.08$^{(4)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$
P. expansum	7.50 ± 0.20$^{(3)\,\mathbf{(1)}}$	0.67 ± 0.03$^{(1)\,\mathbf{(2)}}$	6.87 ± 0.12$^{(5)\,\mathbf{(1)}}$	0.23 ± 0.16$^{(2)\,\mathbf{(2)}}$	6.50 ± 0.10$^{(5)\,\mathbf{(1)}}$	0.27±0.10$^{(2)\,\mathbf{(2)}}$
P. griseofulvum	5.53 ± 0.08$^{(4)\,\mathbf{(1)}}$	0.75± 0.00$^{(5)\,\mathbf{(2)}}$	3.87 ± 0.25$^{(2)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	3.83 ± 0.38$^{(2)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$
P. minioluteum (a)	11.87 ± 0.06$^{(5)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	2.45 ± 0.10$^{(6)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	2.35 ± 0.05$^{(6)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$
P. minioluteum (b)	12.77 ± 0.18$^{(6)\,\mathbf{(1)}}$	0.25± 0.00$^{(7)\,\mathbf{(2)}}$	2.43± 0.28$^{(6)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	2.27 ± 0.08$^{(7)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$
P. nalgiovense (a)	3.93 ± 0.19$^{(7)\,\mathbf{(1)}}$	0.30±0.00$^{(8)\,\mathbf{(2)}}$	5.28 ± 0.24$^{(4)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	5.47 ± 0.19$^{(3)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$
P. nalgiovense (b)	5.13 ± 0.12$^{(8)\,\mathbf{(1)}}$	0.28± 0.08$^{(9)\,\mathbf{(2)}}$	7.08 ± 0.10$^{(1)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	7.25 ± 0.09$^{(8)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$
P. pinophilum	8.87 ± 0.08$^{(9)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	1.23 ± 0.25$^{(7)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	1.12 ± 0.21$^{(9)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$
P. puberulum	5.77 ± 0.12$^{(10)\,\mathbf{(1)}}$	1.43± 0.38$^{(2)\,\mathbf{(2)}}$	5.67± 0.42$^{(3)\,\mathbf{(1)}}$	0.32 ± 0.16$^{(2)\,\mathbf{(2)}}$	5.40 ± 0.26$^{(3)\,\mathbf{(1)}}$	0.28±0.06$^{(2)\,\mathbf{(2)}}$

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
P. aurantiogriseum	1.26$^{(1)}$	1.23$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$
P. brevicompactum	1$^{(2)}$	1$^{(2)}$	1.25$^{(2)\,\mathbf{(1)}}$	1.24$^{(2)\,\mathbf{(1)}}$	1.11$^{(2)\,\mathbf{(2)}}$	1.10$^{(2)\,\mathbf{(2)}}$
P. chrysogenum	1.32$^{(1)}$	1.32$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$
P. corylophilum	1$^{(2)}$	1$^{(2)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$
P.expansum	1.41$^{(3)}$	1.38$^{(3)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$
P. griseofulvum	1.27$^{(1)}$	1.28$^{(1)}$	1.04$^{(3)\,\mathbf{(1)}}$	1.05$^{(3)\,\mathbf{(1)}}$	1$^{(1)\,\mathbf{(2)}}$	1$^{(1)\,\mathbf{(2)}}$
P. minioluteum strain a	1$^{(2)}$	1$^{(2)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$
P. minioluteum strain b	1$^{(2)}$	1$^{(2)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$
P. nalgiovense strain a	1.23$^{(1)}$	1.20$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$
P. nalgiovense strain b	1$^{(2)}$	1$^{(2)}$	1.08$^{(4)\,\mathbf{(1)}}$	1.07$^{(4)\,\mathbf{(1)}}$	1$^{(1)\,\mathbf{(2)}}$	1$^{(1)\,\mathbf{(2)}}$
P. pinophilum	1.22$^{(1)}$	1.20$^{(1)}$	1.05$^{(3)\,\mathbf{(1)}}$	1.05$^{(3)\,\mathbf{(1)}}$	0$^{\mathbf{(2)}}$	0$^{\mathbf{(2)}}$
P. puberulum	1.63$^{(4)}$	1.59$^{(4)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$	1$^{(1)}$

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
P. nalgiovense strain b	0.00	non toxic
P. minioluteum strain b	0.51	non-toxic
P. nalgiovense strain a	1.44	non-toxic
P. brevicompactum	2.21	non-toxic
P. puberulum	6.90	non-toxic
P. griseofulvum	18.64	slightly toxic
P. aurantiogriseum	19.26	slightly toxic
P. pinophilum	23.57	slightly toxic
P. expansum	33.73	slightly toxic
P. chrysogenum	61.95	highly toxic
P. minioluteum strain a	71.48	highly toxic
P. corylophilum	74.67	highly toxic

% of mortality = 100 - (n.º of alive treated brine shrimp larvae /n.º of alive control brine shrimp larvae) x 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 ºC, 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 ºC 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 ºC nor 25 ºC. 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 α-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 δ-(L-α-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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