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Surface mycobiota of home-made dry cured sausages from the main producing regions of Argentina and morphological and biochemical characterization of *Penicillium nalgiovense* populations

Graciela Vila[a,c,*], Juan A. Segura[c], Vanesa Ludemann[a], Graciela N. Pose[a,b,c]

[a] Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes (UNQ), Roque Sáenz Peña 352, B1876BXD Bernal, Provincia de Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, C1033AAJ CABA, Argentina
[c] Universidad Nacional de Río Negro (UNRN), Mitre 331, 8336 Villa Regina, Río Negro, Argentina

ABSTRACT

The characteristics and quality of home-made dry cured sausages can be recognized and associated with the region of origin. The characteristics of this type of sausages result from the superficial mycobiota that spontaneously colonizes the products. The aim of this study was to identify the house mycobiota associated with home-made dry cured sausages from different localities of Argentina and characterize the populations of *Penicillium nalgiovense* present by morphological and biochemical markers. To this end, 79 samples were collected from 10 localities of three main producing regions (Buenos Aires, Córdoba and La Pampa provinces). A total of 196 isolates belonging to six genera and 17 species were obtained. The predominant genus was *Penicillium* (134 of the isolates) and the predominant species was *P. nalgiovense* (108 isolates). The isoenzyme patterns of α-esterase (α-EST; EC 3.1.1.1) and Malate dehydrogenase $NADP^+$(MDHP; EC 1.1.1.40) were characterized in 48 of these isolates (ten from Colonia Caroya, ten from Oncativo, ten from Tandil, nine from Mercedes and nine from La Pampa). A total of 26 bands were observed: 17 for α-EST and 9 for MDHP. α-EST was the most polymorphic isoenzyme, whereas MDPH presented no polymorphism. The results were subjected to numerical analysis. Cluster analysis revealed the formation of two groups: Group I formed by 24 isolates from Córdoba and Buenos Aires provinces and Group II with 24 isolates from La Pampa and Buenos Aires province. These data suggest the existence of morphological and biochemical variations among *P. nalgiovense* populations with different geographical origin.

1. Introduction

During the ripening and drying process of dry cured meat products, molds grow on their surface. These molds contribute to the development of specific flavors and aromas through proteolytic and lipolytic activities. These molds also have an antioxidant effect, maintain a favorable microclimate around the product, protect against pathogenic or spoilage microorganisms and give dry sausages their typical appearance (López-Diaz et al., 2001; Ludemann et al., 2004a, 2004b; Sonjak et al., 2010).

The surface mycobiota of home-made products is mainly composed of environmental fungi (Iacumin et al., 2009; López-Diaz et al., 2001), so the characteristics of these products depend on the place of origin. This mycobiota may be composed of genera and species of both desirable and undesirable filamentous fungi, the latter of which produce not only imperfections in color, odor or taste (Canel et al., 2013), but also mycotoxins (Iacumin et al., 2009; López-Diaz et al., 2001; Ludemann et al., 2009). Thus, several studies have been conducted to identify and characterize the mycobiota and mycotoxin-producing molds present on the surface of dry sausages, especially in countries with a long tradition in their production, such as Italy and Spain (Comi et al., 2004; Marziano et al., 2000), and in other parts of the world (Mintzlaf et al., 1972; Savic and Savic, 2002; Toldrá and Hui, 2015).

In Argentina, few works have studied the surface mycobiota of fermented dry sausages. In a previous study, we analyzed the superficial mycobiota present on artisanal and commercially produced salami with the aim to evaluate the quality and safety of these products (Ludemann et al., 2004b), whereas, in another work, we studied the toxicogenic potential of the *Penicillium nalgiovense* strains isolated (Ludemann et al., 2004a, 2009). Other researchers have also performed studies on dry sausages from Tandil, Buenos Aires province (Castellari et al., 2010) and Colonia Caroya, Córdoba province (Canel et al., 2013).

Although the surface mycobiota of home-made dry cured sausages is heterogeneous, numerous authors have reported that the genus *Penicillium*, in particular *P. nalgiovense*, is very frequent on the surface of salami (Andersen, 1995; Asefa et al., 2010; Castellari et al., 2010; Papagianni et al., 2007). The presence of *P. nalgiovense* on the casings of home-made sausages gives them a homogeneous appearance and

* Corresponding author at: Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes (UNQ), Roque Sáenz Peña 352, B1876BXD Bernal, Provincia de Buenos Aires, Argentina.

E-mail addresses: grav071965@gmail.com (G. Vila), jsegura@unrn.edu.ar (J.A. Segura), vludemann@unq.edu.ar (V. Ludemann), npose@unq.edu.ar (G.N. Pose).

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different colors (white, turquoise, bluish green). These morphological characteristics are used to establish intraspecific differences in *P. nalgiovense* strains according to the predominant biotype (Fink-Gremmels et al., 1988). However, the use of morphological criteria to characterize strains of *P. nalgiovense* from different locations is difficult because their phenotype varies depending on the environmental and geographical conditions.

Determining the characteristics of the house mycobiota in each region is very important for producers in any part of the world because the characteristics and quality of their salami are recognized and associated with the region of origin and because the particular superficial mycobiota that colonizes these products contributes to their identity.

Biochemical markers, such as isoenzymes, allow the characterization of populations independently of the environmental influence. These markers have been used in numerous studies to establish both interspecific variability (Balesdent et al., 1992; Banke et al., 1997; Boshoff et al., 1996; Hepper et al., 1988; Leuchtmann and Keith, 1990; Nicoletti et al., 2008; Petrunak and Christ, 1992; Somé and Tivoli, 1993; Trujillo et al., 2005) and intraspecific variability (Balesdent et al., 1992; Bosland and Williams, 1986; Cameleyre and Olivier, 1993; Faure-Raynaud et al., 1991; Grondona et al., 1997; Nygaard et al., 1989; Zhu et al., 1987) of fungal populations. The advantage is that isoenzymes are the result of direct expression of the genome of an individual and are not influenced by environmental factors (Bhuvanendra et al., 2010; Garber, 1973; Micales et al., 1986; Tiwari et al., 2011).

To our knowledge, morphological markers combined with biochemical markers, such as isozymes, have not been previously used for the intraspecific characterization of *P. nalgiovense* isolates obtained from the superficial mycobiota of home-made sausages from Argentina or other parts of the world. Thus, the objective of this study was to identify the house mycobiota associated with fermented dry sausages from different Argentinean production areas and to characterize the populations of *P. nalgiovense* of each locality by using morphological and biochemical markers.

2. Materials and methods

2.1. Sampling, isolation and counting of molds

A total of 79 samples of home-made dry fermented sausages (salami) were obtained from 33 factories located in 10 localities of the provinces of Buenos Aires, Córdoba and La Pampa, Argentina (Table 1). No fungal starter cultures had been used for the manufacture of these sausages. The sausages sampled had more than 20 days of fermentation ripening time. The diameter and length of the salami were measured.

The molds on the surface of the sausages were isolated according to Canel et al. (2013). Briefly, casings were aseptically removed using sterile scalpels, placed into sterile bags containing 200 ml sterile 0.1% peptone water, and homogenized in a Stomacher for 1 min. From spore suspension, serial dilutions was obtained and 0.1 ml was inoculated in Petri dishes containing Dichloran Glycerol Agar 18% (DG18) or Malt Extract Agar (MEA) to enumerate xerophilic and total culturable fungi, respectively. The cultures were incubated at 25 °C for 7 days. For counting, plates containing 10–100 colonies were used and the results were expressed as colony-forming units per cm^2 of sample (CFU/cm^2).

The relative isolation frequency (Fr) and relative density (Dr) of each genus/species were determined as follows (Gonzalez et al., 1995; Greco et al., 2012):

$$\text{Fr}(\%) = (\text{number of samples with a genus or species/total number of samples})\ 100.$$

$$\text{Dr.}(\%) = (\text{number of isolates of a genus or species/total number of isolates})\ 100.$$

All the isolates were preserved on agar slants of MEA at 4 °C and cryopreserved in 18% glycerol at −80 °C.

2.2. Mold identification

2.2.1. Mold identification based on morphological characters

Filamentous fungi were identified to genus level, according to macro- and microscopic criteria in accordance with Samson et al. (2004). Representative colonies of each type were transferred for subculturing onto plates with MEA.

Fungal isolates were then identified to species level according to Pitt and Hocking (2009). Thus, the isolates were cultured on MEA, Czapek Yeast extract Agar (CYA), 25% Glycerol Nitrate and Creatine Sucrose Nitrate according to the scheme proposed by the authors.

The different biotypes of *P. nalgiovense* were determined according to Fink-Gremmels et al. (1988). The isolates were divided into six biotypes based on colony diameters after 7 days on MEA, conidial color and degree of sporulation on CYA and MEA.

2.2.2. Mold identification based on DNA sequences

Thirty-one isolates morphologically identified as *P. nalgiovense* were randomly selected to confirm the identity at the molecular level. Mycelia of 7-day-old cultures on CYA were collected from their Petri-dishes by scraping the surface of the plates and stored at −80 °C until use. The genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. The genomic DNA was quantified with the fluorometer Qubit 2.0 (Life Technologies) (Greco et al., 2015). The internal transcribed spacer (ITS) of nuclear ribosomal DNA was amplified using the primers ITS1 (5′-TCCGTAGG

Table 1
Fungal count from sausages (CFU/cm^2) in DG18 and MEA media.

Province	Locality	Number of samples studied	Average fungal counts (CFU/cm^2)				
			Total mycobiota		*Penicillium* spp.	Penicillium nalgiovense	Others genus
			DG18	MEA	MEA	MEA	MEA
Buenos Aires	Mercedes	8	4.2×10^7	2.9×10^7	1.2×10^7	1.1×10^7	1.7×10^7
	Tandil	15	$3{,}9 \times 10^8$	4.5×10^7	$1{,}3 \times 10^7$	1×10^7	3.2×10^7
Córdoba	Oncativo	17	1.2×10^8	4.7×10^7	3.2×10^7	3.1×10^7	1.5×10^7
	Colonia Caroya	30	3.6×10^8	4×10^7	1.6×10^7	1.4×10^7	2.4×10^7
La Pampa	Colonia Barón	1	1.6×10^7	1.6×10^7	4.6×10^6	< 10	$1{,}14 \times 10^7$
	General Campos	2	1.8×10^7	$5{,}9 \times 10^7$	4.2×10^7	4.2×10^7	1.7×10^7
	Santa Rosa	1	1.5×10^7	1.6×10^7	1.5×10^7	1.5×10^7	1×10^6
	Jacinto Arauz	2	6.9×10^5	3.92×10^5	3.6×10^5	3.6×10^5	$3{,}2 \times 10^4$
	Alpachiri	1	5.7×10^7	$4{,}6 \times 10^7$	3×10^7	< 10	$1{,}6 \times 10^7$
	Embajador Martini	2	4.6×10^6	1.3×10^6	1×10^6	1×10^6	3×10^5

Count values of surface mycobiota, *Penicillium* spp., *P. nalgiovense* and others genus isolates expressed in CFU/cm^2.

TGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). PCR amplifications were carried out in volumes of 20 μl containing 1× of Taq buffer, 1 μM of each primer, 0.2 mM of dNTPs, 1.5 mM of MgCl2, 1 U of Taq DNA polymerase (Fermentas, International Inc.) and 25 ng of genomic DNA. The PCR amplification protocol consisted of an initial denaturation step of 3 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min. The PCR reactions were performed in a Multigene Thermal Cycler (Labnet International, Inc.). Amplification products were separated by electrophoresis through 1.5% agarose gels with 3 μl of GelRed for visualization. Gels were viewed under UV light and documented on a UVITEC gel documentation computer-based system (Cambridge, UK). The PCR amplicons were sequenced by Macrogen Inc. (Seoul, Korea). The sequences obtained from the different isolates were aligned using the multiple alignment algorithm MUSCLE (Multiple Sequence Comparison by Log-Expectation) and compared to the ITS gene sequences in the NCBI GenBank database by BLAST, and the *P. nalgiovense* ITS gene sequence was downloaded (CBS 352.48). The DNA sequences of our isolates and the sequences downloaded from GenBank were re-aligned using MEGA 7.0 software. Phylogenetic analysis was performed using maximum likelihood (ML) phylogenetic methods, based on the Tamura 3-parameters model. *Aspergillus niger* NRRL 62637 and *P. glabrum* FJ904924.1 were used as outgroups. Sequences from nearby species, *P. dipodomyis* (CBS 110412) and *P. chrysogenum* (CBS 306.48), were add a tree to support the confirmation of identity.

2.3. Isoenzymatic characterization of P. nalgiovense isolates

Forty-eight strains, including all locations, were randomly selected for this assay.

2.3.1. Enzyme systems used

The enzyme systems α-Esterase (α-EST; EC 3.1.1.1), Malate dehydrogenase NADP$^+$ (MDHP; EC 1.1.1.40), Glutamate Oxalacetate Transferase (GOT; EC 2.6.1.1), Superoxide dismutase (SOD; EC 1.15.1.1) and Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) were screened. α-EST and MDHP gave a good resolution and were thus used for the following studies.

2.3.2. Sample preparation

Shake flasks (500 ml) containing 200 ml growth medium (30 g sucrose, 4 g KNO_3, 0.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4$, 5 g extract of yeast, 1 g K_2 HPO_4 in 1 l distilled water) were inoculated with spore suspensions of the cultures at a concentration of 1.10^6 CFU/ml. The fungi were grown for 7 days at 25 °C on a rotation table (150 rpm) (Banke et al., 1997).

After incubation, the mycelia were harvested by filtration under vacuum and washed several times in distilled water before preserved at −20 °C. Then, 1 g of the mycelium harvested was disrupted in a mortar with 2 ml of 0.1 M potassium phosphate buffer (pH 7.0) and the homogenates were centrifuged at 14,000 rpm for 20 min at 4 °C. The concentration of solubilized protein in the supernatants was estimated by the Bradford method by using a Bio-Rad Protein Assay kit (Bio-Rad), using microtiter plates and bovine serum albumin as a standard according to the manufacturer's protocol. Samples were stored at −80 °C until isozyme analysis.

2.3.3. Electrophoresis

Enzymes were separated in a vertical discontinuous non-denaturing polyacrylamide gel electrophoresis (PAGE), according to Laemmli's (1970) method. Samples were adjusted to 1 mg/ ml of protein in 25% glycerol and 0.1% Bromophenol. Then, 20 μl of each sample were loaded on the gel. The resolving gel contained 8–10% polyacrylamide (30 g acrylamide, 0.8 g *N′N′*-bis-methylene-acrylamide), whereas the stacking gel contained 5% polyacrylamide. A 1.5 M Tris-HCl buffer with pH 8.8 was used in the resolving gel, whereas 0.5 M Tris-HCl buffer with pH 6.8 was used in the stacking gel. The reservoir buffer contained 25 mM Tris and 192 mM glycine, pH 8.3. Gels were run at 120 V and 30 mA at 4 °C. The running time was 90–120 min depending on the isoenzyme.

2.3.4. Enzyme staining

The enzymes studied were stained according to the protocols recommended by Murphy et al. (1996), as follows: α-EST was stained by immersion of the gels in a solution containing 50 ml 0.2 M Tris-HCl pH 7.0, 0.05 g fast blue BB salt and 3 ml α-naphthyl acetate solution (stock 1% solution in 50% acetone); MDHP was stained by immersion of the gels in a solution containing 50 ml 0.2 M Tris-HCl pH 8.0, 1 ml 0.1 M $MgCl_2$, 5 ml 2.0 M DL-malic acid pH 8.0, 0.02 g of nicotinamide adenine dinucleotide phosphate, 1 ml of 5 mg/ml nitroblue tetrazolium chloride and 1 ml 5 mg/ml 5-methylphenazinium methyl sulfate. Gels were incubated at room temperature in the dark until the bands were visible.

2.3.5. Data analysis

All the gels were observed and diagrammed immediately after enzyme staining. The relative mobility (Rf) of each enzyme band was calculated (Somé and Tivoli, 1993). Polymorphisms were scored for the presence (1) or absence (0) of bands. Similarity matrices were generated according to Sorensen or DICE coefficients and used to perform cluster analysis using the unweighted pair group method analysis (UPGMA) with the NTSYS-PC 2.1 program. Dendrograms that indicate the estimated similarity among isolates were constructed with the TREE program of NTSYS-PC (Ipek et al., 2003).

3. Results and discussion

As mentioned, fungal counts on dry sausages from different Argentine provinces were determined on two culture media: DG18 and MEA. The average count of xerophilic fungi in DG18 medium ranged from 105 to 108 CFU/cm^2, whereas that in MEA medium ranged from 105 to 107 CFU/cm^2. The highest counts of xerotolerant fungi in DG18 were observed in samples from Córdoba and Buenos Aires provinces. Except the localities of Colonia Barón and Alpachiri in the province of La Pampa, where P. nalgiovense was not found, this species represents more than 77% of the total count of the genus Penicillium (Table 1).

A total of 79 samples were tested and 196 isolates of filamentous fungi were obtained. These fungi belonged to six genera and 17 species. All of them were the result of spontaneous colonization of the casing by the house mycobiota. The genera and species isolated in each locality, as well as their frequency (Fr) and relative density (Dr) are shown in Table 2.

The analysis by province showed that the localities of Córdoba and Buenos Aires provinces showed greater diversity of fungal species than those of La Pampa province. The genus *Penicillium* was determined in 78 out of the 79 samples (Fr 98.73%). *Mucor* was the next most frequently isolated genus (Fr 44.3%). This genus was not isolated in Mercedes (Buenos Aires province). The least frequent genera were *Cladosporium*, *Aspergillus*, *Scopulariopsis* and *Geotrichum*.

P. nalgiovense achieves a desirable external appearance such as color, texture or flavor, whereas the growth of other species may represent health risks due to the production of mycotoxins. This has been reported by numerous researchers (Grazia et al., 1986; López-Diaz et al., 2001; Ludemann et al., 2009; Sorensen et al., 2008). In the present study, *P. nalgiovense* was the most prevalent species of the genus *Penicillium* in all the provinces studied. However, in the localities of Colonia Barón and Alpachiri in the province of La Pampa, *P. nalgiovense* was not isolated, and *P. implicatum* and *P. solitum*, which are considered non-toxicogenic species (Samson and Pitt, 2013), were found with a frequency of 100% (Table 2). Potentially toxicogenic *Penicillium* species were found in all other locations with a frequency of < 40%. These

Table 2
Frequency and relative density of filamentous fungi isolated from sausages of the three Argentine provinces studied.

			Córdoba				Buenos Aires				La Pampa											
Genus	Species	Number of isolates	Caroya n = 30		Oncativo n = 17		Tandil n = 15		Mercedes n = 8		Colonia Barón n = 1		General Campos n = 2		Santa Rosa n = 1		Jacinto Arauz n = 2		Alpachiri n = 1		Embajador Martini n = 2	
			Fr(%)	Dr(%)	Fr(%)	Dr(%)	Fr(%)	Dr(%)	Fr(%)	Dr(%)	Fr(%)	Dr(%)	Fr(%)	Dr(%)	Fr(%)	Dr(%)	Fr(%)	Dr(%)	Fr(%)	Dr(%)	Fr(%)	Dr(%)
Penicillium	*P. nalgiovense*	108	90	76	82	49	67	47	75	44	-	-	100	83	100	67	100	50	-	-	100	50
	P. implicatum	9	-	-	6	4	-	-	50	19	100	25	-	-	-	-	-	-	-	-	-	-
	P. chrysogenum	9	-	-	-	-	40	20	13	6	-	-	-	-	-	-	-	-	-	-	-	-
	P. solitum	3	-	-	-	-	7	3	-	-	-	-	-	-	-	-	-	-	100	67	-	-
	P.brevicompactum	2	7	3	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-
	P. citrinum	2	-	-	6	2	7	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	P. griseofulvum	1	-	-	-	-	7	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mucor	*M. racemosus*	15	27	12	12	4	40	18	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M.circinelloides	12	-	-	47	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	50
	M. hiemalis	7	3	2	6	2	-	-	-	-	100	25	100	17	100	33	100	50	100	33	-	-
	M. piriformis	1	3	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus	*A. ochraceus*	11	-	-	12	6	-	-	62	25	-	-	-	-	-	-	-	-	-	-	-	-
Cladosporium	*C. cladosporoides*	5	-	-	18	6	-	-	13	3	100	25	-	-	-	-	-	-	-	-	-	-
	C.sphaerospermum	2	-	-	-	-	-	-	13	3	100	25	-	-	-	-	-	-	-	-	-	-
	C. herbarum	1	-	-	-	-	7	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Scopulariopsis	*S. candida*	6	7	5	6	4	7	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Geotrichum	*G. candidum*	3	-	-	6	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Fr: relative frequency.
Dr: relative density.

Table 3
Macromorphological characteristics of *Penicillium nalgiovense* colonies. These have been grown in medium MEA and CYA for 7 days at 25 °C.

Province	Localities	Number of isolates	Predominant biotype	Macromophological characters of the predominant biotype						Others biotypes
				Diameter (mm)		Conidium color		Reverse color		
				CYA	MEA	CYA	MEA	CYA	MEA	
Córdoba	Caroya	43	4	26–33	12–18	Turquoise White	Turquoise, Grey greenish, White	Pale-yellow	Orange	1,3,5,6
	Oncativo	25	4	25–30	12–20	Turquoise White	Grey green, White	Yellow	Orange	6
Buenos Aires	Tandil	16	4	25–33	20–25	Turquoise	Turquoise, Grey greenish, White	Pale-yellow	Orange	6
	Mercedes	14	1	20–28	20–25	Turquoise	Greenish turquoise	Pale-yellow	Orange	3,4,5
La Pampa	Colonia Barón General Campos Santa Rosa Jacinto Arauz Alpachiri Embajador Martini	10	1	20–26	18–22	Turquoise Green	Greenish turquoise	Pale-yellow	Orange	3,4,5

Table 4
Biotypes of *Penicillium nalgiovense* isolated from sausages.

Province	Localities	Total isolates	Biotype									
			1		3		4		5		6	
			N° isolates	Dr[a] (%)	N°. isolates	Dr[a] (%)	N° isolates	Dr[a] (%)	N° isolates	Dr[a] (%)	N° isolates	Dr[a] (%)
Córdoba	Caroya	43	1	2	1	2	29	68	3	7	9	21
	Oncativo	25	0	0	0	0	22	88	0	0	3	12
Buenos Aires	Tandil	16	0	0	0	0	11	69	0	0	5	31
	Mercedes	14	9	64	3	22	1	7	1	7	0	0
La Pampa	Colonia Barón	1	0	0	0	0	1	100	0	0	0	0
	General Campos	3	1	50	0	0	0	0	2	50	0	0
	Santa Rosa	2	1	50	1	50	0	0	0	0	0	0
	Jacinto Arauz	2	1	50	0	0	1	50	0	0	0	0
	Alpachiri	0	0	0	0	0	0	0	0	0	0	0
	Embajador Martini	2	1	50	1	50	0	0	0	0	0	0

[a] Isolate relative density.

included: *P. chrysogenum* (which produces roquefortine C, PR toxin, penicillin and secalonic acids), *P. brevicompactum* (which produces mycophenolic acid), *P. citrinum* (the main producer of citrinin), and *P. griseofulvum* (which produces patulin, cyclopiazonic acid, roquefortine C and griseofulvin).

Yellow aspergilli were also found (Aspergillus section Circumdati). The morphological characteristics are similar to those described for *A. ochraceus* and *A. westerdijkiae*. However, a difference of *A. westerdjkiae*, *A. ochraceus* grows at 37° in CYA. In our study the isolates grow at 37 °C in CYA which allows us to confirm that they were *A. ochraceus*. (Visagie et al., 2014b) (Table 2).

These results are consistent with research carried out in different countries of South America, in which the predominant genus found in the surface mycoflora of sausages was *Penicillium* (Canel et al., 2013; Castellari et al., 2010; Galvalisi et al., 2012; Ludemann et al., 2004a, 2004b; Pose et al., 2004) In Europe, numerous works have reported diverse species of the genus *Penicillium* as predominant, including *P. nalgiovense*, *P. solitum* and *P. olsonii* (Andersen, 1995; Asefa et al., 2010; Iacumin et al., 2009; López-Diaz et al., 2001; Papagianni et al., 2007; Sonjak et al., 2010; Sorensen et al., 2008; Tabuc et al., 2004), the latter of which was not isolated in our work.

The presence of mycotoxins in dried meat products is a global health concern (Alapont et al., 2014; Escher et al., 1973; Mintzlaf et al., 1972; Nuñez et al., 1996) and the mycotoxigenic capacity of isolates obtained from dry cured meat products has been demonstrated (Alapont et al., 2014; Iacumin et al., 2009; López-Diaz et al., 2001; Tabuc et al., 2004). The presence of ochratoxin in low concentration has been demonstrated in salami samples from Italy (Armorini et al., 2016; Cabañes, 2000; Dall'Asta et al., 2010; Iacumin et al., 2011; Monaci et al., 2005).

Certain species of fungi can affect not only the safety, but also the quality of dry-cured meat products. Canel et al. (2013) showed that *Aspergillus ochraceus* can produce an undesirable yellowish gold color in the casing of dry fermented sausages, causing important economic losses to producers. These authors isolated *A. ochraceus* from samples from Colonia Caroya (Córdoba) with a frequency higher than 80% in summer. They proposed that very hot summers, when the temperature is high and the a_w of the product decreases, favor the development of *A. ochraceus* on the surface of the salami. In our study, *A. ochraceus* was determined in Mercedes (Buenos Aires province), with a low Dr (5.9%), and in Oncativo (Córdoba province), with a moderate Dr (25%) (Table 2).

With respect to *Penicillium nalgiovense*, colonies with visibly different morphological appearance were isolated. Several biotypes were determined (Table 3). In the two localities of Córdoba province, the predominant biotype was biotype 4. Colonia Caroya presented a greater diversity of biotypes (5). These results are in agreement with those of Canel et al. (2013). In Buenos Aires province, biotype 4 was also predominant in Tandil, whereas biotype 1 was predominant in Mercedes, where a greater variety of biotypes (4) was also determined (Table 4). These were dissimilar respect to previous studies made by Castellari

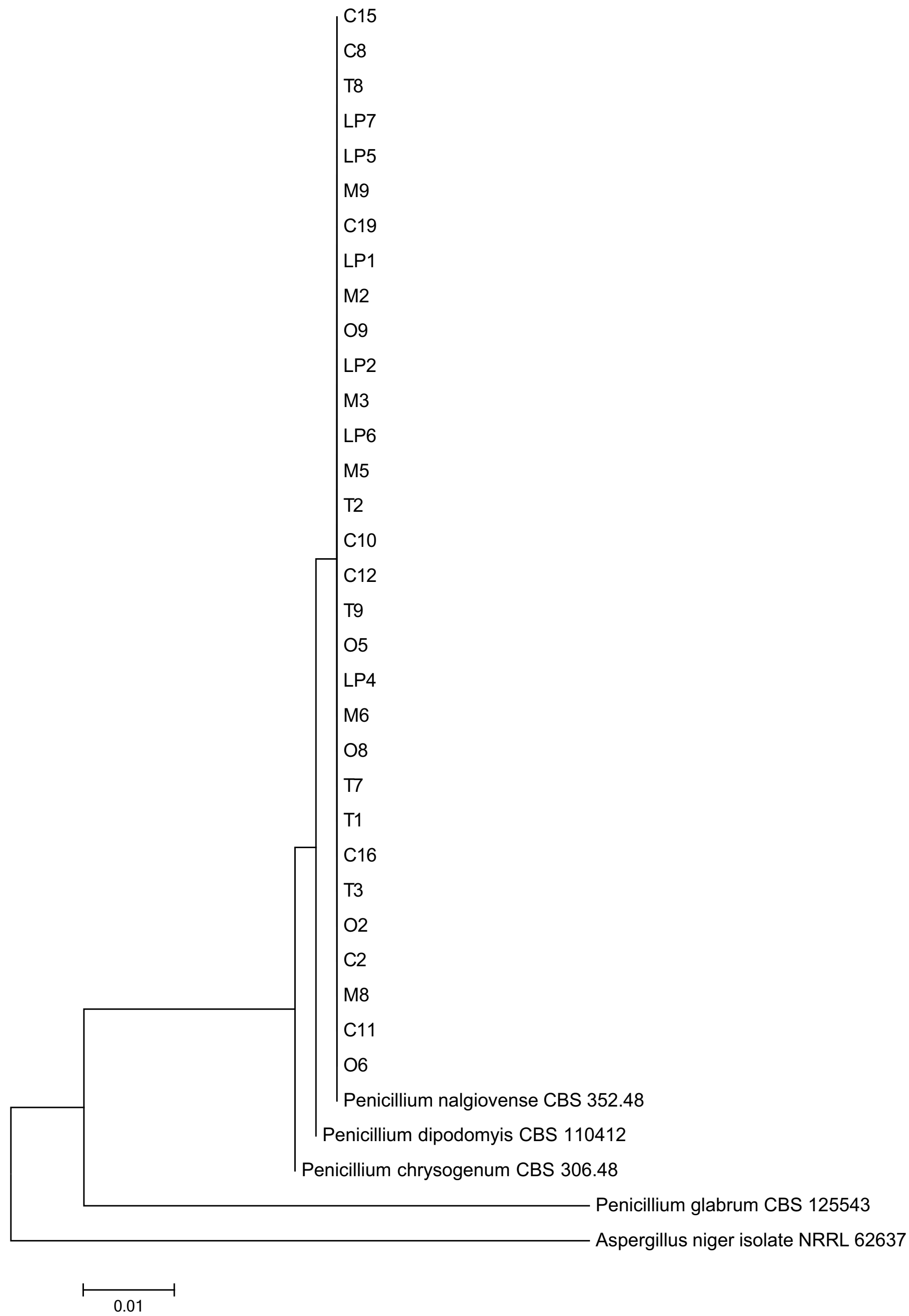

Fig. 1. Dendrogram based on phylogenetic analysis of de ITS1-5, 8S-ITS4 rDNA sequences showing the relationships among 31 randomly selected isolates from P. nalgiovense. Outgroups taxa were included. Maximum likelihood (ML) was used.

et al. (2010), who found biotype 1 in most of the isolates obtained from samples from small artisanal processing factories from Tandilia system. In La Pampa province, biotype 1 was the most frequent in the six localities sampled. The house mycobiota on the surface of fermented sausages in this province had not been previously investigated.

In addition, the 5.8S ITS rDNA regions of a total of 31 isolates of *P. nalgiovense*, identified according to their morphological characters, were amplified and sequenced, and the sequences used to confirm the identity of the isolates. A single fragment of approximately 550 nucleotides was obtained. The size of the ITS sequence obtained agrees with that reported for other species of the same genus (Dupont et al., 1999; Skouboe et al., 1999; Tiwari et al., 2011).

The phylogenetic relationship between the selected isolates was inferred from the neighbor binding tree analysis of aligned ITS sequences. Our results show 100% homology with the reference strain suggested by ICPA for *Penicillium nalgiovense* (Ex-type CBS 352.28) (Visagie et al., 2014a). As expected, the selected strains show little difference in their sequences with species closely related to *P. dipodomyis* (CBS 110412), both belonging to the same group. The analysis of the ITS sequences together with the morphological characteristics of the isolates allowed us to confirm that it is *P. nalgiovense*. No differences were observed between the isolates belonging to different localities or biotypes (Fig. 1).

To study the intraspecific variability in the indigenous populations of *P. nalgiovense*, we analyzed two isoenzymatic systems: α-EST and MDHP. Each isolate presented a characteristic band pattern that we used to determine the variation between populations of different localities.

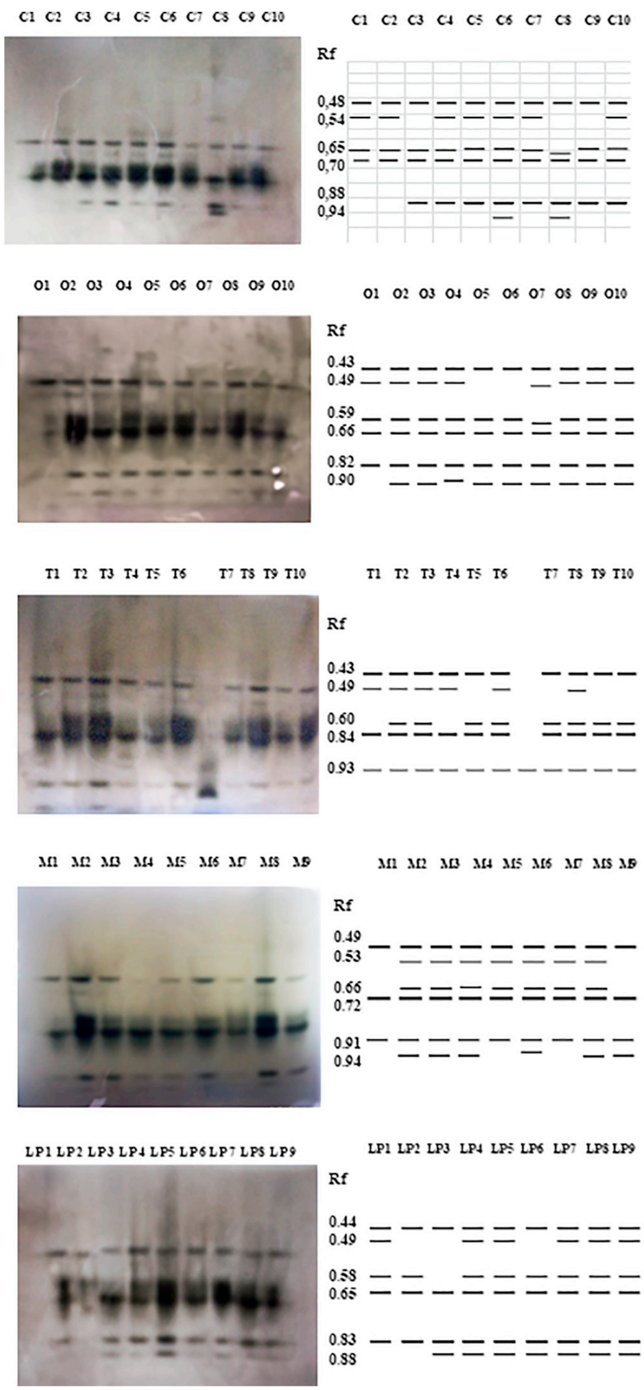

Fig. 2. Schematic representation of the α-EST banding patterns obtained from zymograms of the following localities: Colonia Caroya (C1 to C10), Oncativo (O1 to O10), Tandil (T1 to T10), Mercedes (M1 to M9) and La Pampa (LP1 to LP9). The electrophoretic mobility (Rf) was measured from the anode.

α-EST showed significant polymorphism (Fig. 2), with a total of seventeen active bands (bands with greater expression), eight of which were polymorphic. For each locality, similar banding patterns, formed by five or six active bands, were observed. Each population of fungi differed in the percentage of polymorphic bands, being Colonia Caroya, Mercedes and La Pampa the localities with greatest polymorphism (50%), and Oncativo the one with the lowest levels of polymorphism (33%). Tandil presented 40% of polymorphism and a polymorphic band of Rf: 0.60 exclusive. These results are in agreement with the high level of variability for esterases previously reported in *Penicillium* (Anné and Peberdy, 1981; Banke et al., 1997; Faure-Raynaud et al., 1991; Nicoletti et al., 2008).

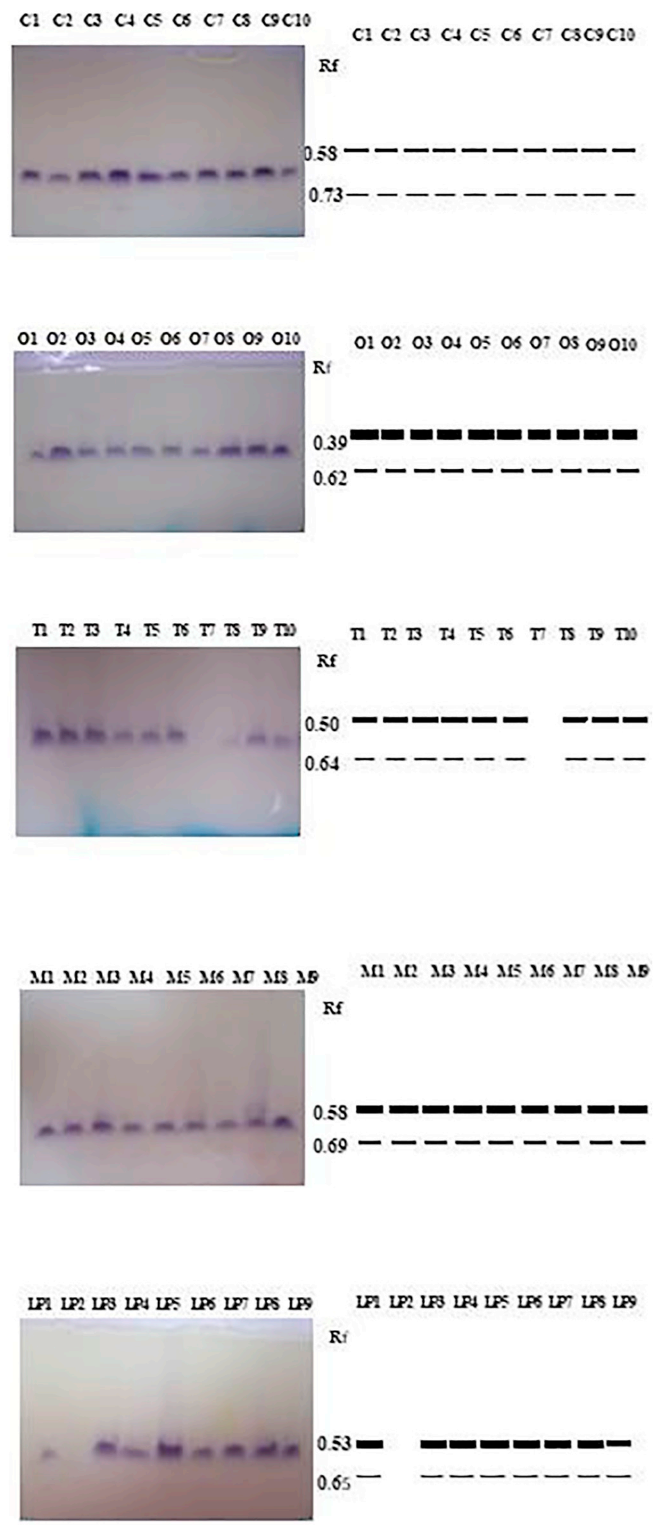

Fig. 3. Schematic representation of MDHP banding patterns obtained from zymograms of the following localities: Colonia Caroya (C1 to C10), Oncativo (O1 to O10), Tandil (T1 to T10), Mercedes (M1 to M9) and La Pampa (LP1 to LP9). The electrophoretic mobility (Rf) was measured from the anode.

The presence of identical or monomorphic bands common to all localities, such as the Rf band: 0.49, consistent with the presence of similar monomorphic bands in isolates from distant localities (e.g. bands of Rf 0.43 and 0.84 in samples from Tandil, General Campos and Oncativo, and bands of Rf 0.65 in samples from Colonia Caroya and

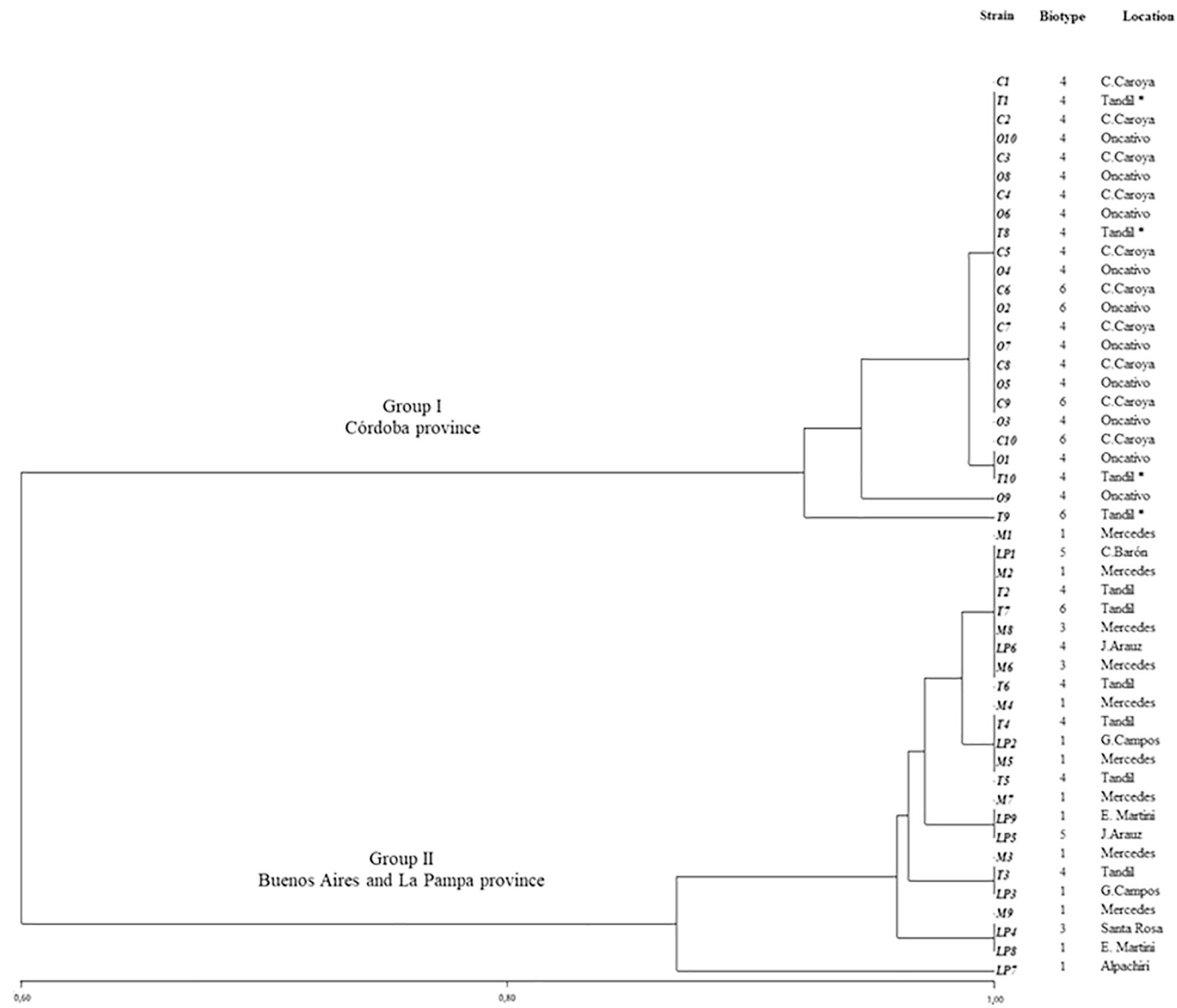

Fig. 4. UPGMA dendrogram illustrating the relationships between the α-EST and MDHP bands of 48 strains of *P. nalgiovense* from Córdoba, Buenos Aires and La Pampa provinces. The DICE coefficient was used.
*Isolates that do not correspond to Córdoba province.

Santa Rosa), suggests that the populations analyzed probably had a common origin and then disseminated, adapting to different environmental conditions (Garber, 1973; Lugo et al., 2001; Zhu et al., 1987).

MDHP showed nine active bands. All of the isolates exhibited the same pattern formed by two active bands with different electrophoretic mobility. Only one isolate from La Pampa showed one band, not two (Fig. 3). Banke et al. (1997) also reported two bands with different electrophoretic mobility, and thus low genetic variability, from *P. chrysogenum* and related species.

The low variability indicates that MDHP is not suitable as biochemical markers to characterize *P. nalgiovense* populations isolated from fermented dry sausages. Monomorphic bands such as those with MDHP may be associated with homology between the populations studied (Garber, 1973; Boshoff et al., 1996; Zhu et al., 1987) and could be used as interspecies markers.

The dendrogram was generated considering the presence or absence of the 26 total bands obtained from the two enzymes analyzed in the 48 selected isolates (Fig. 4).

The cluster analysis showed two groups. Group I included 24 isolates, 20 isolates from Colonia Caroya and Oncativo (Córdoba province) and four isolates from Tandil (Buenos Aires province), 19 of which (79%) corresponded to biotype 4, with the remaining five isolates corresponding to biotype 6. Group II included other 24 isolates (the remaining isolates from Buenos Aires province and all the isolates from La Pampa province). In this group, the biotypes were diverse, although biotype 1 was the predominant one (50%) (Fig. 4).

The cluster analysis allowed observing that the geographical location of the isolates was interpolated and more clearly observing the separation between the *P. nalgiovense* populations from Córdoba province and those from Buenos Aires and La Pampa provinces For other fungi, different researchers have demonstrated intraspecific differentiation by geographical location and host (Hepper et al., 1988; Hwang et al., 1986; Leung and Williams, 1986; Sidaoui et al., 2017; Zhu et al., 1987). However, our results differ from other studies in which it was not possible to relate the site of isolation to the isoenzyme pattern (Boshoff et al., 1996; Bosland and Williams, 1986; Cameleyre and Olivier, 1993; Moreno et al., 2008).

4. Conclusions

In this study, we identified and characterized the dominant genera and species from the surface of dry fermented sausages (salami) in different producing regions of Argentina. Identifying and characterizing the house mycobiota of each producing region around the world is extremely relevant to generate new knowledge as well as to select strains that guarantee a safe and quality product, without altering the properties that provide the unique characteristics to the product in each region (Santa et al., 2014). The presence of altering and/or mycotoxicogenic fungi was shown in this study.

Penicillium nalgiovense generally predominates on the surface of dry fermented sausages. Many studies have evaluated the morphological and biochemical characteristics of this fungus and its influence on the maturation process of salami. However, there are no studies to establish the existence of variability among the populations of *P. nalgiovense* that colonize the surface of the salami in different producing regions. In this study, the congruence between the predominant biotype, isoenzymatic patterns and geographical origin of the isolates confirmed the validity and utility of the use of markers to establish the variability between populations. Isoenzyme electrophoresis was shown to be useful to establish intraspecific differences between study populations and to estimate the genetic relationship between them.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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