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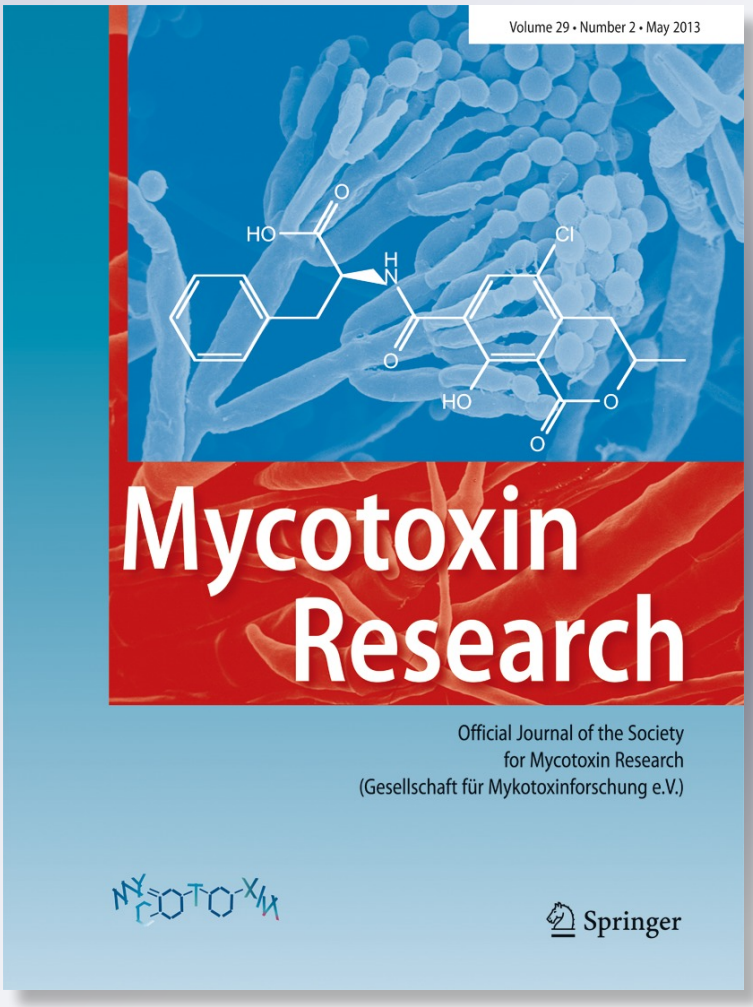

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ORIGINAL PAPER

Gliotoxinogenic *Aspergillus fumigatus* in the dairy herd environment

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Abstract The potential association between hygienic conditions in the environment of lactating cows and the presence of gliotoxinogenic *Aspergillus fumigatus* strains was studied. Milk samples (individual cow's milk [ICM], bulk tank milk [BTM]) from 44 dairy farms were sampled. In ICM samples, eight different species of *Aspergillus* were identified. *A. flavus* and *A. fumigatus* were predominant, with 37.8 % and 26.1 % relative densities, respectively. *A. fumigatus* strains were isolated from 61.4 % of the BTM samples, and 34 % of these strains were able to produce gliotoxin. Principal component analysis was used to associate the presence of *A. fumigatus* with some hygienic and sanitary practices. A significant and positive correlation was observed between dry cow therapy and forestripping. The presence of *A. fumigatus* gliotoxin producers in milk was associated with high somatic cells count (SCC) samples. Good hygienic and sanitary practices were associated with absence of *A. fumigatus* and relatively low SCCs of <250,000 cells/ml. In general, a high percentage of dairy farms were positive for *A. fumigatus* in BTM samples. This is the first work that indicates the positive effects of adequate hygienic and sanitary practices in dairy herds on the control of *A. fumigatus* and related species. By reducing the frequency of *Aspergillus* spp. in the dairy environment, the risk of farm handlers' exposure and the risk of intramammary fungal infections would also be reduced.

Keywords *Aspergillus fumigatus* · Gliotoxin · Somatic cell count · Milk hygiene · Dairy cow

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Introduction

Several species within the genus *Aspergillus* are pathogenic agents, and *A. fumigatus* is one of these. *A. fumigatus* is ubiquitous in the environment, and may cause severe infectious diseases in humans and in animals (Denning 1998; Fischer et al. 2006). In dairy animals, *A. fumigatus* causes mastitis, respiratory infections, abort, and other diseases, the bibliographical references being more numerous for cattle than for sheep and goats (Austwick 1965; Ainsworth and Austwick 1973; Gourreau et al. 1988; Smith 1989).

Mastitis is one of the most important health problems in bovine dairy herds. Experimentally infection assays with *A. fumigatus* in sheep and goat, verified that it can provoke abortion and mastitis (Corbel et al. 1980; Muñoz et al. 1989; Mandal and Gupta 1994; El-Naggar et al. 1997). In addition, several authors described clinical cases of bovine mastitis caused by *A. fumigatus* (Thompson et al. 1978; Schällibaum et al. 1980; Pepin 1988; Bauer et al. 1989; Katamoto and Shimada 1990). Several factors suggest that these outbreaks of mammary aspergillosis are associated with an incorrect or unhygienic (intramammary) administration of antibiotics during drying-off. Contamination of the teat end or cannulas

by environmental *A. fumigatus* could favor further penetration into the mammary gland. The inhibition of bacterial growth by the implementation of antibiotics for mastitis treatment and prevention, may promote the growth of the fungi into the mammary gland, thus influencing the pathogenesis and severity of the intramammary infections (Jensen et al. 1996; Aller et al. 1999; Perez et al. 1999; Las Heras et al. 2000). In addition, *A. fumigatus* spores are easily spread in the air and pose a high risk of exposure for both animals and humans (Land et al. 1987). This fungus is able to produce thermogenic mycotoxins with immunosuppressive, genotoxic, cytotoxic and apoptotic effects, and to induce neurological syndromes in farm workers who have manipulated mouldy feed (Gordon et al. 1993; Nieminen et al. 2002; Upperman et al. 2003; Pereyra et al. 2008). *A. fumigatus* is the most common cause of invasive human and animal aspergillosis, an important source of morbidity and mortality in immunocompromised hosts (Sutton et al. 1996). Dairy herd handlers, who are exposed extensively to this fungus, often develop hypersensitivity, so that they develop severe allergic reactions to the mold. Aspergillosis includes invasive, inflammatory, granulomatous, narcotising disease of lungs, and other organs; and rarely, systemic and fatal disseminated disease (Denning 1998; Denning et al. 2002). Kwon-Chung and Sugui (2009) analyzed the role of gliotoxin in the pathobiology of *A. fumigatus*. This mycotoxin has been suspected as one of the most likely virulence determinants among various secondary metabolites produced by the species. Gliotoxin is a dipeptide characterized by the presence of a disulfide bridge across the piperazine ring. The disulfide bridge allows the cross linking with cysteine residues in proteins and the generation of deleterious reactive oxygen species (ROS) through a redox cycle between the reduced and oxidized forms. This mechanism of ROS generation is believed to be responsible for the toxicity of gliotoxin (Gardiner et al. 2005). A survey of patients of a cancer center in the United States reported a frequency of gliotoxin production of 93 % among clinical *A. fumigatus* isolates (Lewis et al. 2005). These results support the hypothesis that gliotoxin production might act in vivo as a virulence factor required to establish *A. fumigatus* infection.

For all these reasons, it is important reduce the number of microorganisms present on the teat end. Prevention is the key to controlling mammary gland infections and to protect the dairy herd handler. Effective control measures for contagious and environmental pathogens involves: teat dipping, dry cow therapy, milking time hygiene (wash and dry the udders before applying the milking unit), predipping, culling and vaccines (National Mastitis Council 2004; Pellegrino et al. 2008; McDougall et al. 2009; Pellegrino et al. 2010). Teat dipping and dry cow therapy form the basis of the prevention programs (Crist and Harmon 1991; Smith and Hogan 1995).

The relation between hygienic and sanitary practices and the presence of *A. fumigatus* gliotoxin producers in bovine milk has not been informed yet. The aim of this work was to study a possible association between gliotoxinogenic *A. fumigatus* in cow's milk and measures related to good dairy management, such as teat dipping, dry cow therapy, forestripping, and cleaning practice.

Materials and methods

Description of the sampling region

The animals concerned belonged to 44 dairy establishments located in Cordoba province, in the central region of Argentina during March to September, 2009 (Fig. 1) (Vissio et al. 2009). Cordoba province produces 37 % of the country's total milk and is the main milk production area with 3,000 dairy establishments (Ministerio de Agricultura, Ganadería y Pesca de la Nación 2009). It includes four dairy regions, Villa Maria basin being the most important, with 50 % of state milk production and 1.5 million ha covered. The region is also an area of intensive agriculture and livestock production, with significant production of cereals, fruits and oilseeds (soybeans, wheat, corn, sunflower, oats, barley, rye). The climate is mild with average temperatures around 10.8 °C and 24.8 °C in winter and summer, respectively. All farms had mechanical milking systems. The selection of the establishments was focused on the geographical location, the number of cows in milking (100–250) and the daily average milk production (10–20 l/cow). Every dairy establishment was visited once during the morning milking. In each herd, between 20 % and 40 % of the animals (breed, Holando-Argentino) in milking (middle milk lactation) were randomly sampled.

Samples were taken in agreement to the instructions recommended by the National Mastitis Council (2004):

Individual cow's milk (ICM) samples (n=901):

The udders were disinfected with ethanol 70 %, dried with individual paper towel and the first jet of milk discarded (forestripping). A pool sample (ICM) of approximately 40 ml milk from the four quarters of every animal was collected in plastic containers with 0.5 ml Azidiol for the determination of the somatic cells count (SCC), and 10 ml in a sterile pipe for fungal isolation.

Bulk tank milk (BTM) samples (n=44):

Additional samples were collected from the farm cooling tank after homogenization of the content. Milking was performed twice daily and fresh raw milk

Fig. 1 Geographical location of dairy establishments in the Villa Maria dairy basin (Cordoba province, Argentina)

was stored in a cooling tank until delivery for further marketing or processing. Aliquots (100 ml) were removed with the aid of a pole with a cup at the end. Subsamples were taken at nine different locations (near the top, middle and bottom of the tank). Pooled samples, collected in sterile flasks, were kept on ice during the transportation from the farm to the laboratory.

All samples were kept at 4 °C and transported to the laboratory until their processing up to but not more than 18 h after the sample capture. On receipt at the laboratory, samples were warmed to 37 °C in a water bath and stirred very gently with a magnetic stirrer to dissolve the fat layer. The samples were then centrifuged for 15 min at 3,000 *g*, the upper fat layer was discarded with the aid of a Pasteur pipette, and defatted milk was filtered through two or more Whatman No. 4 (Schleicher & Schuell, Dassel, Germany) filter papers. At least 60 ml of each skimmed milk sample was collected and frozen (−20 °C) for subsequent analysis.

Hygienic and sanitary practices

During animal sampling, the implementation of different hygienic and sanitary practices in the dairy establishments was registered as present or absent in order to evaluate its relation to the presence of *A. fumigatus* gliotoxin producer strains. The reported practices were: *teat dipping* (the dipping of all teats after each milking with a sanitizing solution), *dry cow therapy* (post-milking teat germicidal dips, generally contain antibiotics, skin conditioners and protective film), *forestripping* (the expelling of four to five squirts of milk from each quarter before milking) and *washing* (the scrubbing of teats and teat ends thoroughly with a paper towel and washing by hand with water).

Somatic cell count determination

The milk SCC is a key measure of milk quality, reflecting the health status of the mammary gland and the risk of non-physiological changes to milk composition. Generally a SCC is considered normal when the cells per milliliter in ICM are <250,000 (Carrillo-Casas and Miranda-Morales 2012). Udders and cooling tank samples were subject to SCC determination. The SCC was performed with a Somacount 300 (Bentley, Chaska, MN, USA) according to the revised protocol of the 148A method C, fluoro-opto-electronic, International Dairy Federation Laboratory (1995). The value of 250,000 cells/ml was considered as the cut-off point. Samples with SCC values ≥250,000 cells/ml were considered to be from animals with inflamed udders, whereas samples with SCC values <250,000 cells/ml were considered to be from healthy animals (Carrillo-Casas and Miranda-Morales 2012).

Fungal microbiota isolation and identification

Total fungal counts of all udder and cooling tank samples were performed on dichloran rose bengal chloramphenicol agar (DRBC). This is a general medium used for estimating total culturable mycobiota (Abarca et al. 1994). Quantitative enumeration of fungal propagules in solid media was done using the surface-spread method. Ten milliliters of each sample were homogenized in 90 ml 0.1 % peptone water solution (10^{-1}) for 30 min in an orbital shaker. Serial dilutions (10^{-2} and 10^{-3}) were made and 0.1-ml aliquots were inoculated in duplicates onto DBRC medium. The plates were incubated for 7–10 days at 25 °C. Only plates containing 10–100 colony-forming units (CFU) were used for counting, with results expressed as CFU per milliliter of sample. On the last day of incubation, individual CFU counts for each colony type considered to be different were recorded.

For the fungal identification, colonies representative of *Aspergillus* were transferred for sub-culturing to tubes containing malt extract agar (MEA). Taxonomic identification of all colonies was achieved through macroscopic and microscopic studies, followed by standard tests which were related to the genera of each particular group of fungi. Fungal species among *Aspergillus* genus were identified according to Klich (2002) and Samson et al. (2000). The results were expressed as isolation frequency (% of samples in which each genera was present) and relative density (% of isolation of each species among strains of the same genera).

Gliotoxin production by *A. fumigatus* isolates

All *A. fumigatus* strains were assayed for gliotoxin production. The strains were grown on YES (sucrose 40 g, yeast extract 20 g, agar 20 g and distilled water to 1,000 ml) plates at 37 °C for 7 days. Three agar plugs were removed from the central area of the colony, weighed and introduced into a small vial. Chloroform (1 ml) was added to each vial, and the sample–solvent mixture was centrifuged for 10 min at 1,252 g. The supernatant was filtered (Titan filtration system, 17 mm, 0.45 μm; Rockwood, TN, USA) and evaporated to dryness under N_2. The residue was redissolved in the mobile phase and used for gliotoxin analysis by HPLC.

Detection and quantification of gliotoxin

Gliotoxin was determined following the methodology proposed by Pena et al. (2010). The HPLC apparatus used for gliotoxin determination was a Perkin Elmer 200 Series HPLC System equipped with an autosampler and UV detection. Briefly, gliotoxin separation was performed at room temperature on a Phenomenex Luna RP C18(2) column (150×4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA) fitted with a C18 guard column using an isocratic mode: 75 % (1 % acetic acid in water) and 25 % acetonitrile. A column washing of 5 min at 95 % of acetonitrile, followed by 5 min of stabilization at the running conditions was performed between chromatographic runs. Detection was done at 254 nm. The standard solutions in mobile phase were prepared from a 5 $mg ml^{-1}$ solution of pure gliotoxin (Sigma-Aldrich, Buenos Aires) in chloroform, after solvent evaporation. The detection limit determined as a rate $s/n=3$ was 0.2 $\mu g g^{-1}$ and the limit of quantification, as a rate $s/n=10$, was 0.9 $\mu g g^{-1}$, where s means signal (intensity of the toxin peak) and n means signal noise.

Statistical analysis

For *A. fumigatus* isolation frequency and relative density data, the analyses were performed by analysis of variance (ANOVA). The test of Least-Significant Difference (LSD) was used to determine the significant differences between means. The interaction between the presence of *A. fumigatus* and hygienic and sanitary practices, such as teat dipping, dry cow therapy, forestripping and washing, was determined using a principal components analysis (PCA). Statistical significance was at $p<0.05$ level. To determine the association between the presence of *A. fumigatus* in milk samples and the SCC, a principal component analysis (PCA) was performed (INFOSTAT 2009).

Results

Milk from 901 ICM samples was mycologically analyzed (Fig. 2). The presence of eight genera of filamentous fungi and yeasts was observed in milk. The yeasts were the predominant fungi isolated from 56.3 % of the samples. Among toxinogenic genera, *Aspergillus* spp. was the most

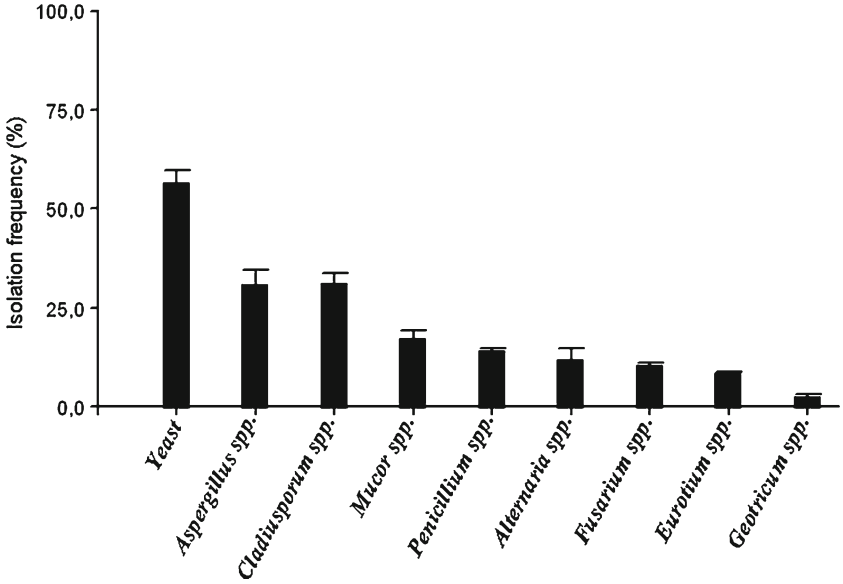

Fig. 2 Isolation frequency (%) of fungal genera from 901 individual cow's milk (ICM) samples

prevalent, followed by *Penicillium* spp, *Alternaria* spp and *Fusarium* spp. *Cladosporium* spp. showed the highest isolation frequency, while *Eurotium* spp and *Geotricum* spp. had the smallest fungal population percentage. Eight species of *Aspergillus* were identified from 901 ICM samples (Fig. 3). *Aspergillus flavus* and *A. fumigatus* were the main isolated species with percentages significantly higher ($p<0.05$) than the other fungal species and significantly different from each other ($p<0.05$). Among toxinogenic species, *A. niger* var *niger* and *A. parasiticus* were also isolated. The other species (*A. terreus, A. niger var. awamori, A. candidus* and *A. foetidus*.) had densities below 10 %, without significant differences among them ($p<0.05$). On the other hand, *A. fumigatus* strains were present in 61.4 % of the BTM samples.

Interaction between *A. fumigatus* and some hygienic and sanitary practices was evaluated using PCA. Principal component 1 (PC1) and principal component 2 (PC2) explained 93.8 % of the total variation of the plot (Fig. 4). PC1 (71.8 %) was associated with dry cow therapy, forestripping and washing, while PC2 (22.0 %) was linked to teat dipping. A significant and positive correlation was observed between dry cow therapy and forestripping ($r=0.70$). Absence of *A. fumigatus* and SCC <250,000 cells/ml were associated to some good hygienic and sanitary practices implemented in the dairy establishments studied (confidence ellipse 95 %). In contrast, presence of *A. fumigatus* and both SCC<250,000 cells/ml or ≥250,000 cells/ml, and absence of *A. fumigatus* with SCC≥250,000 cells/ml were not associated with the practices studied.

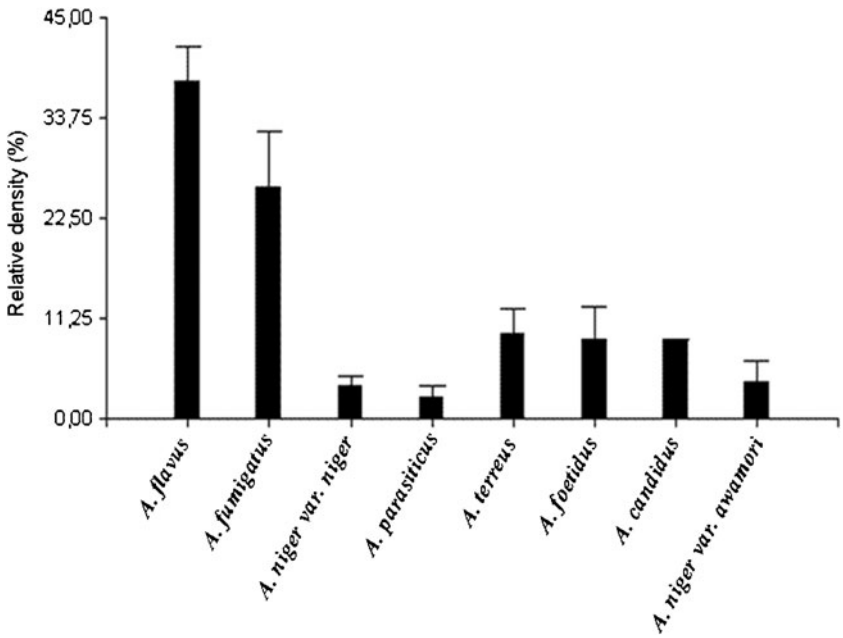

Fig. 3 Relative density (%) of *Aspergillus* species from 901 individual cow's milk (ICM) samples

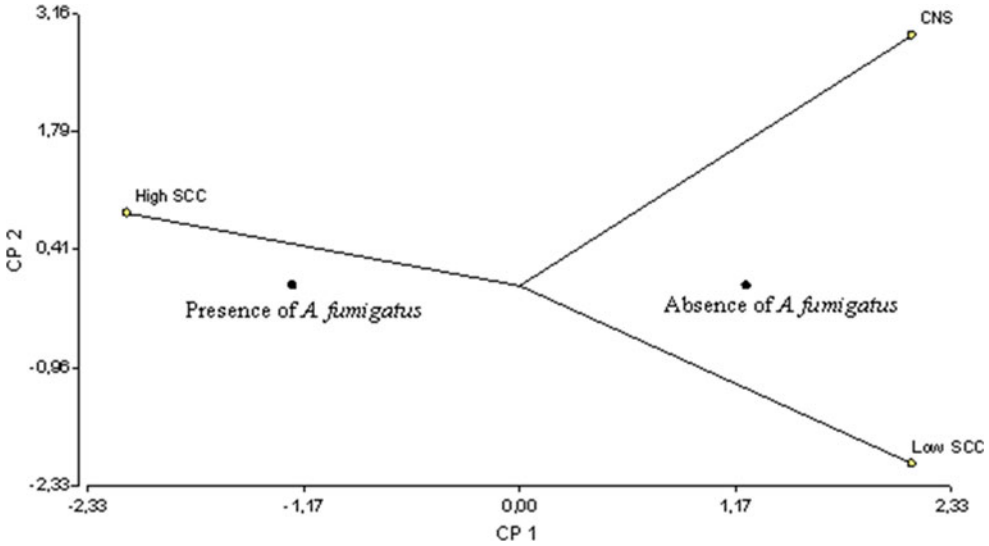

Fig. 4 Principal component analysis of some hygienic and sanitary practices (teat dipping, dry cow therapy, forestripping and washing) in the 44 dairy establishments

The results from SCC determination showed that 75 % (33/44) of the sampled establishments had SCC≥250,000 cells/ml in the bulk tank milk. *Aspergillus fumigatus* were isolated in 60 % of BTM samples with high SCC (20/33). To allow a more detailed analysis, these samples were divided into stratus and the percentage of *A. fumigatus* associated with the different status was evaluated (Table 1). Although there were not significant differences between the presence of *A. fumigatus* and SCC among the different stratus ($p>0.05$), the highest *A. fumigatus* percentage was associated with the highest SCC.

The production of gliotoxin by *A. fumigatus* strains and their relation with SCC is shown in Table 2. Fifteen percent of *A. fumigatus* strains isolated from ICM samples with low SCC (<250,000 cells/ml) were able to produce gliotoxin, whereas 29 % of them isolated from milk with high SCC produced gliotoxin. These strains showed average levels from not detected (ND) to 1.8 $\mu g g^{-1}$. The most productive strains were isolated from the same dairy establishment and were also associated with high SSC.

Discussion

In this study, the presence of *A. fumigatus* strains and their relation with some hygienic and sanitary practices were studied. Moreover, the gliotoxin production and SCC were also evaluated.

Until now, the fungal biota present in milk samples has not been reported. Although yeasts were the prevalent fungi, the main toxigenic genera were also isolated. The high percentage of yeasts isolated could be indicating their capacity to growth in the ducts and acini of the udder, by virtue of their ability to break up into small vegetative-reproducing units. In a previous study, Alonso et al. (2009) described the mycobiota present in raw materials and cow feed from the same selected establishments in the present study and the results indicated that *Aspergillus* was the prevalent genera in cotton seeds, ready cattle feed and corn silage, whereas *Fusarium* spp. were predominant in corn grains. Yeasts and *Penicillium* spp. were present in all kind of samples. Fungi are dispersed throughout the cow's environment, whether hygiene measures are not suitable for the dairy herd animals, the contamination of milk by toxinogenic fungi could occur. Moreover, the presence of fungi alone could predict the milk contamination with mycotoxins. *Cladosporium* spp. showed the highest isolation

Table 1 Relationship between *Aspergillus fumigatus* and somatic cell count (*SCC*) in bulk tank milk samples

SCC (cells/ml)	Percentage (%)/number of samples positive for *A. fumigatus*
>750,000	39.3/13
750,000–500,000	26.3/9
500,000–250,000	34.4/11

Table 2 Gliotoxinogenic *A. fumigatus* strains and somatic cell count (*SCC*)

Aspergillus fumigatus		
SCC (cells/ml)	Gliotoxin producer strains[a]	Gliotoxin levels ($\mu g g^{-1}$)±SD[b]
<250,000	2/13	<QOL
≥250,000	8/28	1.27±0.3

Detection limit of the technique: 0.2 $\mu g g^{-1}$

QOL limit of quantification: 0.9 $\mu g g^{-1}$

[a] Number of producer strains vs total strains

[b] Mean levels ± standard deviation

frequency. Although it does not represent a toxicological risk, it gives indications of environmental contamination levels that affect milk.

A. fumigatus was among the main isolated species from milk samples. This high percentage of *A. fumigatus* isolated from milk samples is of concern because this fungus is able to produce gliotoxin, a toxin that has potent immunosuppressive, genotoxic, cytotoxic and apoptotic effects (Nieminen et al. 2002; Upperman et al. 2003). Pereyra et al. (2008) found strains able to produce more than one mycotoxin and the strains isolated from cattle feed produced higher gliotoxin levels than those isolated from corn silage.

The obtained results showed a high percentage of dairy farms with *A. fumigatus* and related species presence in milk tank samples compared with the low number present in individual cow's milk. This isolation difference was associated with the absence of some basic management and hygiene procedures (teat dipping, washing, dry cow therapy and forestripping) that help to reduce the presence of this fungus in the milk tank. *Aspergillus fumigatus* spores are easily spread in the air and pose a high risk of exposure for both animals and humans (Land et al. 1987).

The milk SCC is a key measure of milk quality, reflecting the health status of the mammary gland and the risk of non-physiological changes to milk composition. Generally an SCC is considered normal when the cells per milliliter are <250,000 (Carrillo-Casas and Miranda-Morales 2012) In this study, an elevated percentage of the establishments (75 %) had a high SCC in their milk tanks. Milk quality is important, with impacts on human health, milk processing and on-farm profitability. High SCC is not associated with direct risks to human health. However, there are a number of indirect risks as a result of poor farm hygiene, antibiotic residues and the presence of pathogenic organisms and toxins in milk.

The most common species of *Aspergillus* that cause invasive aspergillosis world-wide are *A. fumigatus*, *A. terreus*, *A. flavus* and *A. niger*. In order to correlate the pathogenic potential of *A. fumigatus* with the ability to produce gliotoxin and to investigate the taxonomic distribution of gliotoxin-producing *Aspergillus* strains among clinical isolates, Kupfahl et al. (2008) studied a total of 158 *Aspergillus* isolates, comprising four different species—*A. fumigatus* (100), *A. terreus* (27), *A. niger* (16), and *A. flavus* (15)—collected from different medical centers (some isolated from probable cases of aspergillosis) and from environmental samples. Gliotoxin was detected in most culture filtrates of *A. fumigatus* of both clinical (98 %) and environmental (96 %) origin. The toxin was also detected, with decreasing frequency, in culture filtrates of *A. niger* (56 %), *A. terreus* (37 %), and *A. flavus* (13 %). The higher gliotoxin concentrations were detected in *A. fumigatus* strains cultures, whereas the gliotoxin productivities of other *Aspergillus* species were significantly lower. Given these findings, only *A. fumigatus* and related species strains were selected in this work to determine the gliotoxin producing ability. In this work, 24 % of *A. fumigatus* were able to produce gliotoxin and were mainly associated with samples with high SCC. The extent of the production of gliotoxin could vary in individual strains of *A. fumigatus*, depending on the culture conditions. The most important finding of this study is that in milk samples some *A. fumiugatus* strains were able to produce gliotoxin. This result leads to the prediction that gliotoxin will be synthesized if adequate conditions are produced. Additionally, the objective of the present work was not to demonstrate the presence of gliotoxin as a virulence factor. However, an elevated percentage of *A. fumigatus* strains were associated with elevated SCC. For all these reasons, reducing the number of fungi that can reach the mammary gland through the use of preventive mediated is important to reduce the contact between dairy herd handlers and toxinogenic microorganisms.

This is the first study to demonstrate that the presence of *A. fumigatus* should be controlled by the implementation of adequate hygienic and sanitary practices in dairy herds. These practices would reduce the risk of farm handler's exposure and avoid the potential contribution of this microorganism to intramammary infections.

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Conflict of interest None.

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