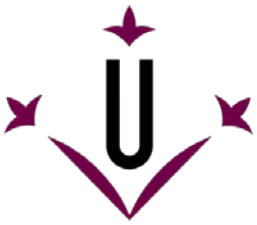

Received Date : 14-Jun-2016
Revised Date : 02-Dec-2016
Accepted Date : 19-Dec-2016
Article type : Original Article

Modelling the effect of pH and water activity in the growth of *Aspergillus fumigatus* isolated from corn silage

V. Alonso [a,b], L. Cavaglieri[a,b]*, A.J. Ramos [c], A. Torres[a,b], S. Marin[c]

[a]Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto. Ruta 36 km 601 (5899) Río Cuarto, Córdoba, Argentina

[b]Member of research career CONICET, Ciudad Autónoma de Buenos Aires.

[c]Food Technology Department, Lleida University, UTPV-XaRTA, Agrotecnic Center, Lleida, Spain.

Running head: Modelling the growth of *Aspergillus fumigatus*

*Corresponding author

Dra. Lilia Rene Cavaglieri

Departamento de Microbiología e Inmunología. Universidad Nacional de Rio Cuarto

Ruta 36 Km 601 (5800) Río Cuarto. Córdoba Argentina.

Phone +54-358-4676429

Fax +54-358-4676231

lcavaglieri@exa.unrc.edu.ar

Abstract

Aims: The aim of this work was to use mathematical kinetic modelling to assess the combined effects of $a_{W,}$ pH, O_2 availability and temperature onto the growth rate and time to growth of *A. fumigatus* strains isolated from corn silage.

Methods and Results: A full factorial design was used in which two factors were assayed: pH and a_W. The a_W levels assayed were 0.80, 0.85, 0.90, 0.92, 0.94, 0.96, 0.98 and 0.99. Levels of pH assayed were 3.5, 4, 4.5, 5, 6, 7, 7.5, and 8. The assay was performed in normal oxygen tension at 25 and 37 °C, and at reduced oxygen tension at 25 ºC. Two strains of *A. fumigatus* isolated from corn silage were used. Kinetic models were built to predict growth of the strain under the assayed conditions. Cardinal ones gave a good quality fit for radial growth rate data. The results indicate that environmental conditions which take place during silage production, while limiting growth of most microorganisms, would not be able to control *A. fumigatus.* Moreover, pH levels in silage, far from limiting its growth, are also close to its optimum. A 5% of CO_2 in the environment did not significantly affect its growth.

Conclusions: A need for a further and controlled acidification of the silage exists, as no growth of *A. fumigatus* was observed at pH 3.5, as long as the organoleptic characteristics of the silage are not much compromised.

Significance and Impact of Study: *Aspergillus fumigatus* is one of the major opportunistic pathogens able to cause illness such as allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis to rural workers. Animal's exposure to *A. fumigatus* spores can result in infections, particularly in those organs exposed to external invasion, as the airways, mammary gland and uterus at birth.

Key Words: Cardinal parameters model, environmental mycology, fungi, predictive modelling, predictive mycology.

Introduction

Aspergillus fumigatus is within the *Aspergillus* spp. species, one of the major opportunistic pathogens able to cause illness such as allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis, depending on the underlying disease and the patient immune condition (Debeaupuis *et al*. 1997). This fungus is isolated from a wide range of substrates including plants, wood, air, cotton seeds, compost, silage and, especially, soil (the main ecological niche growing on decaying organic matter), and its main ecological role is to recycle organic carbon and nitrogen through the environment (Millner *et al*. 1977, Wilson *et al.* 2002). In recent decades, this fungus has become more important as an opportunistic pathogen mainly due to the increasing immunosuppressive diseases. It produces a large number of secondary metabolites, such as gliotoxin, fumiclavins, fumitoxins, verruculogen, fumitremorgens, fumagillin, helvolic acid, tripacidin, esfingofungins, aurantin, fumiquinazol, that have proven to be highly toxic (genotoxic, carcinogenic, immunosuppressant, apoptotic) to both human and animal (Sabater-Vilar *et al*. 2003, Khoufache *et. al*. 2007). *A. fumigatus* is also able to produce a large amount of extracellular enzymes (elastases, phospholipases, serine proteases, aspartic proteases, metalloproteases, cellulases, glucuronidases) that allow it, along with other factors, to survive in adverse and varied environments (Rementeria *et al*. 2005).This great enzymatic capacity and toxicogenic potential, together with its nutritional versatility and thermotolerance enables it for a great pathogenicity. Conidia are able to germinate at temperatures above 37 ºC and the activation and expression of genes that involve

germination have been ascertained (Debeaupuis *et al*. 1997). Moreover, it is able to grow in environments with high concentrations of CO_2 and N_2, with limited nutrients and oxidative stress *A. fumigatus* conidia are resistant to both physical and chemical agents, and tend to remain in air due to their small size (2-3.5 µm) and hydrophobicity. It is estimated that the human being inhales about 200 *A. fumigatus* viable conidia every day, and for people working with compost or in barns, the amounts to which they are exposed may be higher.

Animal's exposure to *A. fumigatus* spores can result in infections, particularly in those organs exposed to external invasion, as the airways, mammary gland and uterus at birth. Respiratory diseases are common in poultry and lead to significant economic losses (Lair-Fuellering *et al*. 2003). *A. fumigatus* has also been isolated from equine nasopharyngeal cavity, producing respiratory diseases in these animals (Guida *et al*. 2005). Various veterinary diseases such as mastitis or placentitis have been described as well as different types of lung infections caused by *A. fumigatus* (Cómera *et al*. 2007) which have been characterized by residual colonization airways or injuries in lung cavity.

A. fumigatus often contaminates silage, hay and cereals for animal consumption causing economic losses due to spoilage and loss of nutritional value thereof, besides representing a serious risk to animal health and to farm workers who manipulate mouldy feed (Boudra *et al*. 2005; El-Shanawany *et al*. 2005). Silage consists in green forage preserved by spontaneous lactic fermentation under anaerobic conditions (Miller 2001). At present this practice is considered one of the most appropriate forms to preserve the nutritional value of animal feed. Several authors have demonstrated the presence of *A. fumigatus* strains and their mycotoxins in hay, silage and different cereals which are consumed by cattle (dos Santos *et al*. 2002). In Argentina and Brazil different researchers found a high density of *A.*

fumigatus strains able to produce gliotoxin, and other tremorgenic mycotoxins in silage and finished feed for dairy cows (Pereyra *et al*. 2008, Pena *et al*. 2009, González Pereyra *et al*. 2011, Keller *et al*. 2012, Alonso *et al*. 2013).

Fungal growth and mycotoxin accumulation in forages depends of multiple environmental factors such as a_W, T and O_2 availability (Bellí *et al*. 2004). For silage, it is also important to determine the influence of pH on fungal growth, since anaerobiosis and pH decrease constitute the preservation strategies in this kind of feed. Corn silage constitutes in Argentina the main basis of cattle diet, and *A. fumigatus* is one of the main fungi causing spoilage. In order to improve the quality and safety of feed and safety of workers, it is necessary to study the effect of these factors in order to predict fungal growth. Besides, prevention of fungal growth effectively prevents mycotoxin accumulation. Although some studies have determined the influence of environmental factors on the growth of *A. fumigatus*, none of them applied mathematical models to quantify these effects (Pena *et al*. 2015, Alonso *et al*. 2015, 2016). Mathematical modelling can be a useful tool to predict and consequently, to prevent the growth of mycotoxicogenic fungi and also to study their response to environmental factors. Some studies have applied secondary kinetic models to modelling growth of aflatoxigenic *A. flavus* (Samapundo *et al*. 2007, Marín *et al*. 2009, García *et al*. 2011, Yue *et al*. 2011, Astoreca *et al.* 2012). However, these models have not been, so far, applied to study *A. fumigatus* growth. In this work, the growth of the same *A. fumigatus* strains used by Alonso *et al.* (2015) was assessed under a number of levels of factors (a_W, pH). Therefore, the aim of this work was to use mathematical kinetic modelling to assess the combined effects of a_W, pH, O_2 availability and temperature onto the growth rate and time to growth of *A. fumigatus* strains isolated from corn silage.

Materials and methods

Experimental design

A full factorial design was used in which two factors were assayed: pH and a_W. The a_W levels assayed were 0.80, 0.85, 0.90, 0.92, 0.94, 0.96, 0.98 and 0.99. Levels of pH assayed were 3.5, 4, 4.5, 5, 6, 7, 7.5, and 8. The assay was performed in normal oxygen tension at 25 and 37 °C, and at reduced oxygen tension (0.4 %O_2 and 5% CO_2) at 25ºC. A CO_2 incubator (Innova-CO48, New Brunswick Scientific, Edison, NJ, USA) was used to create the modified atmosphere. Colony diameters were recorded along the time under each condition. Three replicates for each treatment were used and the study was repeated twice.

Fungal isolates

Two strains of *A. fumigatus* (RC031 and RC032) isolated from corn silage used in the production of cattle feeds in Argentina were used in this study. These isolates were kept in the National University of Río Cuarto, Córdoba, Argentina (RC) Collection Centre. They had previously been characterized as gliotoxin producers (Pellegrino *et al*. 2013).The silage samples were collected from a feed plant located in the south of Córdoba Province in 2011.

Media

Silage extract agar medium (SEM) was made by boiling 30 g of fresh corn silage in 1 L of distilled water for 45 min. The volume was made up to 1 L and 2% agar was added. The pH of the medium was modified with hydrochloric acid (1 mol l^{-1}) and sodium hydroxide (1 mol l^{-1}) corroborated by pH meter. The a_W in the media was modified by the addition of known amounts of glycerol to reach 0.80, 0.85, 0.90, 0.92, 0.94, 0.96, 0.98 and 0.99. Representative samples of each medium were checked with AquaLab Series 3 equipment

(Decagon Devices, Inc., WA, USA). Additionally, control plates were prepared and measured at the end of the experiment in order to detect any significant deviation of a_W.

Inoculation and incubation conditions

Plates for each treatment were centrally inoculated using 5 µL of a fungal spore suspension harvested from 7-day-old cultures on malt extract agar (MEA) using glycerol solutions adjusted to the appropriate a_W for each treatment. The suspensions were shaken and diluted to obtain a suspension of 10^5 spores mL^{-1} adjusted using a Neubauer chamber. Inoculated Petri dishes of the same a_W were sealed in polyethylene bags and incubated for 25 days.

Growth assessment and model fitting

A two-step modelling approach, including primary and secondary modelling was employed. Two perpendicular diameters of the growing colonies were measured daily (mm) until the colony reached the edge of the plate. The diameters (D) of the colonies were plotted against time and a nonlinear regression was applied to estimate the maximum growth rate (μ_{max}, mm/day), latency prior mycelium proliferation (λ, day) and maximum colony diameter (D_{max}), if applicable, by fitting the experimental data to the primary model of Baranyi and Roberts (1994) by using Statgraphics® Plus version 5.1 (Manugistics, Inc, Maryland, USA).

$$D = \mu \max A - \ln\left\{1 + \frac{[exp(\mu maxA) - 1]}{exp(Dmax - D)}\right\}$$

$$A = t + \left(\frac{1}{\mu max}\right)\ln[exp(-\mu maxt) + exp(-\mu max\lambda) - exp(-\mu maxt - \mu max\lambda)]$$

(1)

Analysis of variance (ANOVA) was applied to μ_{max} and λ data in order to establish the significance of the assayed factors (a_W, pH, T, CO_2). The combined effects of pH and a_W on the radial grow rate were determined according to the cardinal secondary models proposed by Rosso *et al.* (1995) and Sautour *et al*. (2001) by multifactorial regression. The model is described by the following equation:

$$\mu max(pH, aw) = 0 \; if \; pH < pHmin \; or \; aw < awmin$$

$$\mu max(pH, aw) = 0 \; if \; pH > pHmax \; or \; aw > awmax$$

$$\mu max(pH, aw) = \mu opt.\rho(pH).\rho(aw) \; if \; pH \; min < pH < pHmax \; and \; awmin < aw < awmax$$

(2)

where

$$\rho(aw) = \left(\frac{(aw - awmin)^2.(aw - awmax)}{(awopt - awmin).[(awopt - awmin)(aw - awopt) - (awopt - awmax)(awopt + awmin - 2aw)]}\right)$$

(3)

$$\rho(pH) = \left(\frac{(pH - pHmin)^2.(pH - pHmax)}{(pHopt - pHmin).[(pHopt - pHmin)(pH - pHopt) - (pHopt - pHmax)(pHopt + pHmin - 2pH)]}\right)$$

(4)

Where $a_{Wmin}$is the a_W below which growth is no longer observed, a_{Wmax} is the a_W above which no growth occurs and a_{Wopt} is the a_W at which maximum growth rate equals its optimal value μ_{opt}. Equally, pH_{min} is the pH below which growth is no longer observed, pH_{max} is the pH above which no growth occurs, and pH_{opt} is the pH at which maximum growth rate equals its optimal value μ_{opt}.

Untransformed data showed stabilised variance, and for this reason Eq. (2) was used with no further transformation of growth rate data.

Results

Initial analysis of the variance of colony diameters as affected by temperature, CO_2, pH, a_w and time for the two *A. fumigatus* strains, showed no significant difference between both repetitions of the assay ($p<0.01$), thus all replicated data were pooled. There was a significant difference between both strains, thus it was decided to work with them separately. In both strains, temperature, pH and a_W had a significant influence ($p<0.05$), while the CO_2 tension did not exert a significant influence on growth, although the interaction with pH and a_W was significant.

Kinetic primary model

Growth of *A. fumigatus* on silage medium followed, in general, a complete Baranyi's function with some exceptions occurring mainly at 37 °C where growth followed a biphasic curve (without upper asymptote) (R^2=0.91-0.94).

Tables 1 and 2 show some μ_{max} and λ estimated through Baranyi's primary model. Regardless of the O_2 tension and temperature levels no growth was observed neither at 0.8 a_W nor at 0.85 a_W. Similarly, no growth was observed at pH 3.5, except at $\geq$0.94 a_W. At the remaining pH levels, in general, there were not significant differences in the growth parameters, thus in Tables 1 and 2 only three levels of pH (4, 6 and 8) are shown. No significant difference in λ ($p>0.05$) was found between 25 and 37 ºC, but significantly higher μ_{max} were observed at 37 ºC ($p<0.05$). It is clear that there is a great extension of latency (λ, lamda) at reduced water activities and higher pHs. Regarding CO_2 concentration

increase, it influenced on λ, leading to a delay in grow initiation, but not on subsequent $\mu_{max.}$.

Secondary modelling for the effects of temperature and pH on the growth rate and time to growth

The cardinal values of environmental factors (minimum, maximum and optimum value) of the secondary cardinal model are shown in Table 3. The two strains showed a similar pattern of behaviour. Model (2) showed a good fit with R^2= 83-91 and MSE= 0.3-2.4, depending on growth conditions and strain.

In the pH, temperature, oxygen tension and a_W range studied the optimal conditions for growth for the assayed *A. fumigatus* strains were 0.99-1 a_w and 4.7-6.6 pH with maximum predicted growth rate of 5.2 mm/day and 5.2-5.7 mm/day at 25 °C with and without reduced O_2 tension, respectively, and 10.3-10.5 mm/day at 37 °C. Estimated $a_{w\ min}$ varied from 0.84 to 0.86. Regarding pH, estimated pH_{min} ranged from 2.2 to 2.3 (Table 3). The combined effect of pH and a_w at the different temperature and CO_2 treatments is shown in figure 1, The most important impact on growth rate was that of temperature, with much faster growth at 37ºC, moreover, the increase in growth rate with a_w is also marked. The pH effect cannot be observed in the figures, since, as explained before, the growth was very similar from pH 4 to 8, while no growth was observed at 3.5. Similarly, the figures were similar for the treatments with or without CO_2, with slightly smaller growth rates under 5% CO_2.

Discussion

The combined effects of CO_2, pH, T and a_w, onto the maximum growth rate of *A. fumigatus* strains isolated from corn silage were assessed in this work.

A silage-based agar medium (SEM) was used to consider nutrients available in the environment of silage. Use of a rich laboratory medium might have probably encouraged *A. fumigatus* growth whereas SEM might be closer to the nutrient levels in real silage ecosystem. Astoreca *et al*. (2009), in a study of kinetic models on *A. flavus* growth proposed that the development of predictive models in rich laboratory media may overestimate the ability of fungi to grow in food, and lead to a predicted unrealistic broad range of growth conditions.

The production of corn silage entails incorporation of the whole plant and its storage is based on the principle of preservation under anaerobic conditions with the growth of lactic acid bacteria. Typically, a_W levels reported in the silages showed values between 0.90-0.99. Gonzalez Pereyra *et al*. (2011) informed, in corn trench silos and silo bags samples in our region, a_W levels from 0.901 to 0.985. These levels were quite homogeneous in all samples (around 0.98), except for samples from lower and upper sections of trench silos, where average values were significantly lower. In corn silage samples from Brazil, Keller *et al*. (2013) informed a_W values that varied from 0.924 to 0.992 showing a mean value of 0.960. According to our results, these conditions are all conducive to *A. fumigatus* growth.

The ensiling process can be divided into different stages: Phase 1, Aerobic phase in which pH is still within the normal range for fresh forage (pH 6.0-6.5). Phase 2, Fermentation phase. Lactic acid bacteria promote a natural fermentation that lowers the pH to a level that is considered unfavourable for the growth of clostridia and most moulds (Richard *et al*. 2007) and pH decreases to 3.8-5.0. Phase 3, Stable phase, for the longest

time in which it can prevent the entry of air, better properties are retained (Merry *et al*. 1997). After silo is opened, the air inlet increases and the pH can rise. According to the mild effect of pH and CO_2 observed in this study on *A. fumigatus*, the preservation strategies may fail in its prevention. Moreover, the silo compaction is of relevant care; when pH is over than 4, *A. fumigatus* growth is optimum. This poses a serious problem in contamination control of silages, since the basis for its preservation is pH decrease to pH 4-5, accompanied by an anaerobic phase. Experimentally CO_2 levels in silage have not been determined. However a fermentative process occurs if it has been proved, so it is assumed that exist CO_2 concentration sufficient for microorganisms deciding to make this anaerobic process instead of one aerobic. Normally to simulate microaerophilic in vitro conditions, standardized 5% CO_2 is used. Although initially CO_2 inhibits microbial growth, when silo is open and oxygen tension increases *A. fumigatus* spores present may start growing, since the a_w levels allow it and low pH may not help controlling it. In relation to the results of our study, the extension of time to growth (λ) indicates that a combination of CO_2 and low pH would help to inhibit *A. fumigatus* growth.

Kinetic models for description of mould growth affected by a_w, were initially proposed by Gibson *et al.* (1994). Samapundo *et al*. (2007) and Mousa *et al*. (2011) considered the combined effect of both a_w and temperature in their models. Marín *et al*. (2012) applied such existing mathematical models to predict the *A. flavus* growth and aflatoxin production in pistachio nuts. Moreover, Rosso *et al*. (1995) proposed a model that takes into account the effect of pH on microbial growth.

Estimated μ_{opt} values were significantly higher at 37 °C than at 25 °C; this is not good in case of an opportunistic pathogen such as *A. fumigatus*. Temperatures variation in silage usually varies between 24 and 25° C (Gonzalez Pereyra *et al*. 2008, Alonso *et al*. 2009),

once silo is opened. Anyway the sense of studying the temperature of 37 °C refers to body temperature. Few studies about the influence of environmental factors, specifically temperature, on the growth of *A. fumigatus* have been informed; Alonso *et al*. (2015) determined the influence of a_W at 25°C on *A. fumigatus* strains considering the prevention of fungal contamination in cattle feed, whereas in another work they demonstrated that these strains were able to grow under similar conditions to human lung (37 °C) and had similar pathogenicity characteristics to clinical strains (Alonso *et al*. 2016). Regarding $a_{w,}$ under suitable a_w conditions (>0.90), *A. fumigatus* strains grew regardless of the pH, temperature or oxygen tension levels tested. Few studies are available on growth parameters of *A. fumigatus*, especially from strains isolated from feedstuff. Pena *et al.* (2015) studied the interaction of temperature, a_w and incubation time on *A. fumigatus* growth and found that at 18 °C and a_w lower than 0.95 *A. fumigatus* growth is inhibited, however these data are not relevant for the silage environment, due to low temperature. Notably it does not exist in literature another work regarding application of mathematical models on *A. fumigatus* growth description, Astoreca *et al.* (2012) model the effects of temperature (10–40 °C) and aw (0.80–0.98), in two media on the growth rates and growth boundaries of three strains of A. flavus isolated from corn in Argentina. They found a good fit to the Rosso cardinal models combined with the gamma-concept which showed that the optimal conditions of growth for the three assayed *A. flavus* isolates on both tested media were 0.98–1 aw and 32–36 °C. Growth limits they could be estimated by applying these models (a_W 0.79 to 0.85 and T° 8.4 to 10.0 °C) are useful for the grain corn conservation, but can´t be extrapolated to the silage system.

The pathogenicity of different *A. fumigatus* strains is variable and there are discrepancies on this point; some authors have suggested that isolates from clinical cases are more

virulent than those isolated from the environment or feed, in terms of gliotoxin production or their enzymatic potential (Lewis *et al.* 2005; Kupfahl *et al.* 2008; Sehnaz and Sevtap 2008). On the other hand, Soleiro *et al*. (2013) determined that isolates from clinical cases and feed do not show significant differences in their molecular analysis. In any case, it must be kept in mind that a higher number of strains would be required for better development of mathematical models applied to *A. fumigatus*.

In conclusion, in this study we showed that environmental conditions which take place during silage production, while limiting growth of most microorganisms, would not be able to control *A. fumigatus.* The existing a_w range allows in any case *A. fumigatus* growth and even may be close to its optimum. Moreover, pH levels in silage, far from limiting its growth, are also close to its optimum. Finally, a 5% of CO_2 in the environment did not significantly affect its growth. A need for a further and controlled acidification of the silage exists, as no growth of *A. fumigatus* was observed at pH 3.5, as long as the organoleptic characteristics of the silage are not much compromised.

Aknowledgements

The authors are grateful to: Consejo Nacional de Ciencia y Técnica (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-FONCYT), Secretaría de Ciencia y Técnica (SECyT-UNRC) and Programa iberoamericano de ciencia y tecnología para el desarrollo (CYTED).

Conflict of Interest

Authors of this paper declare that there is no conflict of interest that should be disclosed about this work

References

Alonso, V., Vergara, L. D., Aminahuel, C., Pereyra, C., Pena, G., Torres, A., Dalcero, A., Cavaglieri, L. (2015) Physiological behaviour of gliotoxigenic *Aspergillus fumigatus* sensu stricto isolated from maize silage under simulated environmental conditions. *Food Addit Contam Part A* **32**(2):236-244.

Alonso, V., Aminahuel, C., Díaz Vergara, L., Pereyra, C., Poloni, V., Dalcero, A., Cavaglieri L. (2016) Ecophysiology of environmental *Aspergillus fumigatus* strains at different a_W and pH conditions. Comparison of gliotoxin production and elastase activity among environmental and clinical strains. *Lett Appl Microbiol* **62** (2), 160-168.

Alonso, V.A., Monge, M.P., Dalcero, A.M., Keller, K., Rosa, C.A.R., Cavaglieri, L.R., Chiacchiera, S.M. (2009) Contribution of raw materials on dairy cattle feedstuff aflatoxin contamination in central Argentina. *Revista Brasilera de Medicina Veterinaria* **31**, 92-99.

Alonso, V.A., Pereyra C. M, Keller, LA.M.,Dalcero, A.M., Rosa C.A.R., Chiacchiera, S.M., Cavaglieri, L.R. (2013) Fungi and mycotoxins in silage: an overview. *J Appl Microbiol* **115**, 637-643.

Astoreca, A., Vaamonde, G., Dalcero, A., Ramos, A.J., Marín, S. (2012) Modelling the effect of temperature and water activity of *Aspergillus flavus* isolates from corn. *Int J Food Microbiol* **156**, 60-67.

Baranyi, J., Roberts, T.A. (1994) A dynamic approach to predicting bacterial growth in food. *Int J Food Microbiol* **23**, 277-294.

Bellí, N., Ramos, A.J., Sanchis, V., Marín, S. (2004) Incubation time and water activity effects on ochratoxinA production by *Aspergillus* section Nigri strains isolated from grapes. *Lett Appl Microbiol* **38**, 72-77.

Boudra, H., Morgavi, D. (2005) Mycotoxin risk evaluation in feeds contaminated by *Aspergillus fumigatus*. *Anim Feed SciTechnol* **120**,113-123.

Coméra, C., André, K., Laffitte, J., Collet, X., Galtier, P., Maridonneau-Parini, I. (2007) Gliotoxin from *Aspergillus fumigatus* affects phagocytosis and the organization of the actin cytoskeleton by distinct signaling pathways in human neutrophils. *Microbes Inf* **9**, 47-54.

Debeaupuis, J.P., Sarfati, J., Chazalet, V., Latge, J.P. (1997) Genetic Diversity among Clinical and Environmental Isolates of *Aspergillus fumigatus. Infect Immun* **65**, 3080-3085.

Dos Santos,V., Dorner, J., Carreira, F. (2002) Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. *Mycopathologia* **156**, 133-138

El-Shanawany, A., Eman Mostafa, M., Barakat, A. (2005) Fungal populations and mycotoxins in silage in Assiut and Sohag governorates in Egypt, with a special reference to characteristic *Aspergilli* toxins. *Mycopathologia* **159**, 281-289.

García, D., Ramos, A.J., Sanchis, V., Marín, S. (2009) Predicting mycotoxins in foods: a review. *Food Microbiol* **26**, 757-769.

Gibson, A.M., Baranyi, J., Pitt, J.I., Eyles, M.J., Roberts, T.A. (1994) Predicting fungal growth: the effects of water activity on *Aspergillus flavus* and related species. *Int J Food Microbiol* **23**, 419-431.

Gonzalez Pereyra, M.L., Alonso, V.A., Sager, R., Morlaco, M.B., Magnoli, CE., Astoreca, A.L., Rosa, C.A.R., Chiacchiera, S.M., Dalcero, A.M., Cavaglieri, L.R. (2008) Fungi and selected mycotoxins from pre- and postfermented corn silage. *J Appl Microbiol* **104**, 1034–1041.

González Pereyra, M.L., Chiacchiera, S.M., Rosa, C.A.R., Sager, R., Dalcero, A.M., Cavaglieri, L.R. (2011) Comparative analysis of mycobiota and mycotoxins contaminating corn trench silos and silo bags. *J. Sci. Food Agric*. **91**, 1474-1481.

Guida, N., Mesplet, M., Di Gennaro, E., Digilio, P., Moras, E.V. (2005) *Aspergillus fumigatus* in nasopharyngeal cavity of horses. *RevIberoamMicol* 22(3)160-162.

Hall, L., Denning, D. (1994) Oxygen requirements of *Aspergillus* species. *J. Med. Microbiol*. **41**, 311-315.

Keller, L., González Pereyra, M., Keller, K., Alonso, V., Oliveira, A., Almeida, T., Barbosa, T., Nunes, L., Cavaglieri, L., Rosa, C. (2013) Fungal and mycotoxins contamination in corn silage: Monitoring risk before and after fermentation. *J Stored Prod Res* **52**, 42-47.

Keller, L.A.M., Keller, K.M., Monge, M.P., Pereyra, C.M., Alonso, V.A., Cavaglieri, L.R., Chiacchiera, S.M., Rosa, C.A.R. (2012) Gliotoxin contamination in pre- and postfermented corn, sorghum and wet brewer's grains silage in Sao Paulo and Rio de Janeiro State, Brazil. *J Appl Microbiol* **112**, 865-873.

Khoufache, K., Puel, O., Loiseau, N., Delaforge, M., Rivollet, D., Coste, A., Cordonnier, C., Escudier, E., Botterel, F., Bretagne, S. (2007) Verruculogen associated with *Aspergillus fumigatus* hyphae and conidia modifies the electrophysiological properties of human nasal epithelial cells. *Microbiol* **23**, 5-7.

Kosalec, I., Pepeljnjak, S., Jandrlíc, M. (2005) Influence of media and temperature on gliotoxin production in *Aspergillus fumigatus* strains. *Arh Hig Rada Toksikol* **56**, 269-273.

Kupfahl, C., Michalka, A., Lass-Flörl, C., Fischer, G., Haase, G., Ruppert, T., Geginat, G., Hof, H. (2008) Gliotoxin production by clinical and environmental *Aspergillus fumigatus* strains. *Int J Med Microbiol* **298**, 319-327.

Lair-Fulleringer, S., Guillot, J., Desterque, C., Seguin, D., Warin, S., Chermette, R. and Bretagne, S. (2003) Differentiation of *Aspergillusfumigatus* isolates from breeding turkeys and their environment by genotyping with microsatellite markers. *J ClinMicrobiol* **41** 1798-1800.

Lewis, R.E., Wiederhold, N.P., Lionakis, M.S., Prince, R.A., Kontoyiannis, D.P. (2005) Frequency and species distribution of gliotoxin-producing *Aspergillus* isolates recovered from patients at a tertiary-care cancer center. *J ClinMicrobiol* **43**, 6120-6122.

Marín, S., Ramos, A.J., Sanchis, V. (2012) Modelling *Aspergillus flavus* growth and aflatoxins production in pistachio nuts. *Food Microbiol* **32**, 378-88.

Marín, S., Colom, C., Sanchís, V., Ramos, A.J. (2009) Modelling of growth of aflatoxigenic*A. flavus* isolates from red chilli powder as a function of water availability. *Int J Food Microbiol* **128**, 491-496.

Marks, B.P. (2007) Status of microbial modelling in food process models. *Comp. Rev. Food Sci. Food Saf* **7**, 137-143.

Merry, R.J., Lowes, K.F., Winters, A. (1997) Current and future approaches to biocontrol in silage. 1727, *in:* Jambor *et al.,* 1997, q.v.

Miller, L.A., Moorby, J.M., Davies, D.R., Humphreys, M.O., Scollan, J.C., Macrae, J.C., Theodorou, M.K. (2001) Increased concentration of water-soluble carbohydrate in perennial ryegrass (*Loliumperenne* L.) Milk production from late lactation dairy cows. *Grass Forages Sci* **56**, 383-394

Millner, P.D., Marsh, P.B., Snowden, R.B., Parr, J.F. (1977) Occurrence of *Aspergillus fumigatus* during composting of sewage sludge. *Appl Environ Microbiol* **34**, 765-772.

Mousa, W., Ghazali, F.M., Jinap, S., Ghazali, H.M., Radu, S. (2011) Modelling the effect of water activity and temperature on growth rate and aflatoxin production by two isolates of *Aspergillus flavus* on paddy. *J ApplMicrobiol* **111**, 1262-74.

Pellegrino, M., Alonso, V., Vissio, C., Larriestra, A., Chiacchiera, S.M., Bogni, C., Cavaglieri L. (2013) Gliotoxinogenic*Aspergillus fumigatus* in the dairy herd environment. *Mycotoxin Res* **29**, 71-78.

Pena, G.A., Pereyra, M., Armando, M.R., Chiacchiera, S.M., Magnoli, C.E., Orlando, J.L., Dalcero, A.M., Rosa, C.A.R., Cavaglieri, L.R. (2010) *Aspergillus fumigatus* toxicity and gliotoxin levels in feedstuff for domestic animals and pets in Argentina. *Lett Appl Microbiol* **50**, 77-81.

Pena, G.A., Monge, M.P., Landa, M.F., Dalcero, A.M., Rosa, C.A.R., Cavaglieri, L.R., (2015) Growth and gliotoxin production by feed-borne *Aspergillus fumigates sensu stricto* strains under different interacting environmental conditions. *World Mycotoxin J* **8** (1) 75-85.

Pereyra, C.M., Alonso, V.A., Rosa, C.A., Chiacchiera, S.M., Dalcero, A.M., Cavaglieri, L.R. (2008) Gliotoxin natural incidence and toxigenicity of *Aspergillus fumigatus* isolated from corn silage and ready dairy cattle feed. *World Mycotoxin J* **1**, 457-462.

Rementeria, A., López-Molina, N., Ludwig, A., Vivanco, A., Bikandi, J., Pontón, Jose., Garaizar, J. (2005) Genes and molecules involved in *Aspergillus fumigatus* virulence. *Rev Iberoam Micol* **22**, 1-23

Richard, E., Heutte, N., Sage, L., Pottier, D., Bouchart, V., Lebailly, P., Garon, D. (2007) Toxigenic fungi and mycotoxins in mature corn silage. *Food ChemToxicol* **45**, 2420-2425.

Rosso, L., Lobry, J.R., Bajard, S., Flandrois, J.P. (1995) Convenient model to describe the combined effects of temperature and pH on microbial growth. *Appl Environl Microbiol* **61**, 610-616.

Sabater-Vilar, M., Nijmeijer, S., Fink-Gremmels, J. (2003) Genotoxicity assessment of five tremorgenic mycotoxins (fumitremorgen B, paxilline, penitrem A, verruculogen, and verrucosidin) produced by molds isolated from fermented meats. *J Food Prot* **66**, 2123-2129.

Samapundo, S., Devlieghere, F., Geeraerd, A.H., De Meulenaer, B., Van Impe, J.F., Debevere, J.(2007) Modelling of the individual and combined effects of water activity and temperature on the radial growth of *Aspergillus flavus* and *A. parasiticus* on corn. *Food Microbiol* **24**, 517-529.

Sautour, M., Dantigny, P., Divies, C., Bensoussan, M. (2001) A temperature-type model for describing the relationship between fungal growth and water activity. *Int J Food Microbiol* **67**, 63-69.

Sehnaz, A., Sevtap, A. (2008) Investigation of extracellular elastase, acid proteinase and phospholipase activities as putative virulence factors in clinical isolates of *Aspergillus* species. *J Basic Microbiol* **48**, 1-7.

Soleiro, C.A., Pena, G.A., Cavaglieri, L.R., Coelho, I., Keller, L.M., Dalcero, A.M., Rosa, C.A.R. (2013) Typing clinical and animal environment *Aspergillus fumigatus*gliotoxin producer strains isolated from Brazil by PCR-RFLP markers. *Lett Appl Microbiol* **57**, 484-491.

Watanabe, A., Kamei, K., Sekine, T., Higurashi, H., Ochiai, E., Hashimoto, Y., Nishimura, K. (2004) Effect of aeration on gliotoxin production by *Aspergillus fumigatus* in its culture filtrate. *Mycopathologia* **157**, 245-254.

Weinberg, Z.G., Muck, R.E. (1996) New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiol Reviews* **19**, 53-68.

Whiting, R.C. (1995) Microbial modelling in foods. *Crit. Rev. Food Sci. Nut*. **6**, 467-494.

Wilson, D.M., Mubatanhema, W., Jurjevic, Z. (2002) Biology and ecology of mycotoxigenic *Aspergillus* species as related to economic and health concerns. *Adv Exp Med Biol* **504**, 3-17.

Yue, X., Sui, J., Niu, T., Liu, Y., Liu, X. (2011) Modeling the effect of temperature and water activity on the growth rate and lag phase of *Aspergillus flavus* during rice drying. *Drying Technol* **29**, 1306-1312.

Table 1. Estimated maximum growth rates (μ_{max}) and time to growth (λ) for *Aspergillus fumigatus* strains at different CO_2, pH and a_W levels, at 25°C.

a_w	pH	*A. fumigatus* RC31		*A. fumigatus* RC31 5% CO_2		*A. fumigatus* RC32		*A. fumigatus* RC32 5% CO_2	
		μ_{max}(mm/day)±SE	λ (day)±SE	μ_{max}(mm/day)±SE	λ (day)±SE	μ_{max}(mm/day)±SE	λ (day)±SE	μ_{max}(mm/day)±SE	λ (day)±SE
0.80	4	-†	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-
0.85	4	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-
0.90	4	0.84±0.01	10.1±0.1	0.91±0.04	1.59±0.15	0.31±0.03	6.49±0.65	md‡	5.34±0.22
	6	0.47±0.03	4.87±0.45	0.96±0.11	5.17±0.21	0.52±0.01	10.82±0.31	0.36±0.04	9.49±0.6
	8	0.41±0.02	11.19±0.25	0.82±0.07	33.33±0.2	md		0.40±0.07	9.59±0.52
0.92	4	0.5±0.02	8.49±0.45	0.56±0.02	2.05±0.33	0.52±0.01	4.3±0.39	0.66±0.05	2.41±0.45
	6	0.56±0.01	4.5±0.31	0.62±0.04	0.54±0.44	0.84±0.02	7.27±0.22	0.84±0.04	4.93±0.31
	8	0.66±0.01	5.36±0.34	0.64±0.07	1.23±1.18	0.50±0.01	3.24±0.39	0.68±0.03	1.05±0.52
0.94	4	1.88±0.07	1.23±0.2	1.58±0.04	1.17±0.18	-	-	1.90±0.11	2.09±0.38
	6	2.08±0.02	2.08±0.1	1.66±0.04	2±0.15	1.80±0.08	2.4±0.29	1.46±0.11	1.55±0.51
	8	0.58±0.01	2.48±0.36	1.85±0.08	2.42±0.31	1.80±0.07	2.8±0.2	1.94±0.04	2.77±0.08
0.96	4	1.33±0.04	1.57±0.35	md	md	md	md	md	md
	6	2.31±0.03	3.52±0.13	2.46±0.07	3.61±0.17	2.62±0.05	2.16±0.17	1.97±0.04	1.23±0.25
	8	2.01±0.03	1.59±0.19	2.36±0.05	3.34±0.12	1.87±0.04	0.99±0.26	2.18±0.05	2.06±0.24
0.98	4	4.61±0.06	0.16±0.07	4.46±0.14	1.41±0.16	3.96±0.11	0.9±0.16	3.67±0.05	0.94±0.08
	6	3.94±0.16	0.29±0.25	3.41±0.09	0.35±0.18	3.77±0.09	0.94±0.15	3.52±0.03	0.96±0.06
	8	5.26±0.09	0.29±0.07	3.48±0.05	0.3±0.1	3.38±0.33	0.91±0.65	3.9±0.04	1.08±0.06
0.99	4	4.88±0.12	0.29±0.12	4.69±0.11	0.98±0.11	5.34±0.13	1.1±0.1	4.71±0.05	0.98±0.05
	6	5.03±0.08	0.29±0.08	4.68±0.1	0.9±0.11	4.42±0.19	0.72±0.22	4.03±0.18	0.87±0.24
	8	5.57±0.15	0.42±0.11	4.12±0.08	1.02±0.11	4.44±0.14	0.82±0.18	4.59±0.04	1.2±0.04

† -, no growth
‡ md, missing data

Table 2. Estimated maximum growth rates (μ_{max}) and time to growth (λ) for *Aspergillus fumigatus* incubated at 37°C with different pH and water activity levels.

a_W	pH	RC31 37ºC		RC32 37ºC	
		μ_{max}(mm/day)±SE	λ (day)±SE	μ_{max}(mm/day)±SE	λ(day)±SE
0.80	4	-†	-	-	-
	6	-	-	-	-
	8	-	-	-	-
0.85	4	-	-	-	-
	6	md‡	md	md	md
	8	md	md	0.38±0.09	-
0.90	4	0.81±0.04	1.51±0.22	-	-
	6	2.92±0.44	2.25±0.15	0.82±0.08	2.79±0.73
	8	1.01±0.12	5.87±0.19	0.71±0.06	5.56
0.92	4	1.07±0.04	4.17±0.34	0.66±0.08	3.31±0.45
	6	0.55±0.02	1.33±0.4	0.43±0.02	5.38±0.83
	8	md	md	md	md
0.94	4	1.69±0.04	0.02±0.17	1.34±0.03	0.46±0.01
	6	2.97±0.11	1.17±0.17	2.31±0.08	0.82±0.23
	8	2.34±0.05	0.84±0.11	0.60±0.03	0.88±0.25
0.96	4	1.81±0.05	0.15±0.34	2.01±0.99	0.4±0.31
	6	2.94±0.07	1.32±0.17	3.79±0.09	0.44±0.14
	8	3.12±0.09	0.21±0.2	4.41±0.13	1.33±0.17
0.98	4	10.5±0.25-	0.25±0.15	8.71±0.31	0.26±0.02
	6	9.83±0.11	0.36±0.01	6.58±0.21	0.55±0.01
	8	6.99±0.01	0.45±0.03	7.85±0.17	0.69±0.04
0.99	4	11.5±0.47	0.15±0.69	11.28±0.16	0.19±0.11
	6	10.66±0.42	0.15±0.58	10.84±0.5	0.41±0.86
	8	7.89±0.17	0.15±0.17	8.68±0.19	0.21±0.15

† -, no growth
‡ md, missing data

Table 3. Estimated values and statistic of the coefficients of the cardinal model for *A. fumigatus* strains at different conditions of temperature a_W and CO_2.

Parameters	RC031			RC032		
	25 °C	**25 °C - 5% CO_2**	**37 °C**	**25 °C**	**25 °C - 5% CO_2**	**37 °C**
μ_{opt}	5.70±0.224	4.69±0.10	11.39±0.33	4.72±0.13	5.15±0.09	8.88±0.27
$a_{W\ min}$	0.85±0.00	0.84±0.01	0.86±0.01	0.85±0.00	0.84±0.01	0.85±0.01
$a_{W\ opt}$	1.00±0.01	1.00±0.12	1.00±0.05	1.00±0.07	1.00±0.09	1.00±0.05
pH_{min}	2.25±0.14	2.28±0.13	2.33±0.14	2.24±0.15	2.20±0.16	2.29±0.12
pH_{max}	11.94±0.61	11.36±0.41	9.65±0.24	10.59±0.52	11.76±0.73	9.99±0.26
pH_{opt}	6.58±0.27	6.20±0.15	5.52±0.11	5.19±0.20	4.75±0.14	5.57±0.11
R2	90.6	88.8	84.7	83.4	90.5	83.0
MSE	0.34	0.30	02.33	0.51	0.26	2.38

RC031 RC032