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

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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 on the growth rate and time to growth of Aspergillus 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. The levels of pH assayed were 3, 3-5, 4, 4-5, 5, 6, 7, 7-5 and 8. The assay was performed at normal oxygen tension at 25°C and at reduced oxygen tension at 25°C. 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. The cardinal models gave a good quality fit for radial growth rate data. The results indicate that the environmental conditions which take place during silage production, while limiting the growth of most micro-organisms, would not be able to control A. fumigatus. Moreover, pH levels in silage, far from limiting its growth, are also close to its optimum. Carbon dioxide at 5% 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 the 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. Exposure of animals to A. fumigatus spores can result in infections, particularly in those organs exposed to external invasion, such as the airways, mammary gland and uterus at birth.

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's 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 humans and animals (Sabater-Vilar et al. 2003; Khoufache et al. 2007). Aspergillus 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 toxigenic potential, together with its nutritional versatility and thermostolerance enables it to have great pathogenicity. Conidia are able to germinate at temperatures above 37°C 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 CO₂ and N₂, with limited nutrients and oxidative stress A. fumigatus conidia are resistant to both physical and chemical agents, and tend to remain in the 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 exposure to A. fumigatus spores can result in infections, particularly in those organs exposed to external invasion, such as the airways, mammary gland and uterus at birth. Respiratory diseases are common in poultry and lead to significant economic losses (Lair-Fulleringer et al. 2003). Aspergillus fumigatus has also been isolated from the 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 (Coméa et al. 2007) which have been characterized by residual colonization airways or injuries in the lung cavity.

Aspergillus 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 and Morgavi 2005; El-Shanawany et al. 2005). Silage consists of green forage preserved by spontaneous lactic fermentation under anaerobic conditions (Miller et al. 2001). At present this practice is considered one of the most appropriate forms for preserving 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 that 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 tremogenic mycotoxins in silage and finished feed for dairy cows (Pereyra et al. 2008; Pena et al. 2010; González Pereyra et al. 2011; Keller et al. 2012; Alonso et al. 2013).

Fungal growth and mycotoxin accumulation in forage depends on multiple environmental factors such as aW, T and O₂ availability (Bell et al. 2004). For silage, it is also important to determine the influence of pH on fungal growth, since anaerobiosis and a pH decrease constitute preservation strategies in this kind of feed. In Argentina corn silage constitutes 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 the safety of workers, it is necessary to study the effect of these factors in order to predict fungal growth. Besides, the 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 the growth of aflatoxigenic Aspergillus flavus (Samapundo et al. 2007; Marín et al. 2009; García et al. 2009; Yue et al. 2011; Astoreca et al. 2012). However, these models have not been, so far, applied to study the growth of A. fumigatus. 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 (aW, pH). Therefore, the aim of this work was to use mathematical kinetic modelling to assess the combined effects of aW, pH, O₂ availability and temperature on 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 aW. The aW levels assayed were 0.80, 0.85, 0.90, 0.92, 0.94, 0.96, 0.98 and 0.99. The levels of pH assayed were 3.5, 4, 4.5, 5, 6, 7, 7.5 and 8. The assay was performed at normal oxygen tension at 25 and 37°C, and at reduced oxygen tension (0.4% O₂ and 5% CO₂) at 25°C. A CO₂ incubator (Innova-CO48; New Brunswick Scientific, Edison, NJ) was used to create the modified atmosphere. Colony diameters were recorded along 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 \(^{a}W_{v}\) 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., Pullman, WA). Additionally, control plates were prepared and measured at the end of the experiment in order to detect any significant deviation of \(^{a}W_{v}\).

**Inoculation and incubation conditions**
Plates for each treatment were centrally inoculated using 5 \(\mu\)l of a fungal spore suspension harvested from 7-day-old cultures on malt extract agar using glycerol solutions adjusted to the appropriate \(^{a}W_{v}\) 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 \(^{a}W_{v}\) 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 diameter \((D)\) of the colonies was plotted against time and a nonlinear regression was applied to estimate the maximum growth rate \((\mu_{\text{max}} \text{ mm per day})\), latency prior to mycelium proliferation \((\lambda, \text{ day})\) and maximum colony diameter \((D_{\text{max}})\), if applicable, by fitting the experimental data to the primary model of Baranyi and Roberts (1994) by using Statgraphics\textsuperscript{®} Plus ver. 5.1 (Manugistics, Inc, Rockville, MD).

\[
D = \mu_{\text{max}}A - \ln \left(1 + \frac{\exp(\mu_{\text{max}}A) - 1}{\exp(D_{\text{max}} - D)}\right)
\]

\[
A = t + \left(\frac{1}{\mu_{\text{max}}}\right) \ln \left[\exp(-\mu_{\text{max}}t) + \exp(-\mu_{\text{max}}\lambda) - \exp(-\mu_{\text{max}}t - \mu_{\text{max}}\lambda)\right] \tag{1}
\]

Analysis of variance was applied to \(\mu_{\text{max}}\) and \(\lambda\) data in order to establish the significance of the assayed factors \((^{a}W_{v}, \text{pH}, T, \text{CO}_{2})\). The combined effects of pH and \(^{a}W_{v}\) on the radial growth 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:

\[
\mu_{\text{max}}(\text{pH},^{a}W_{v}) = 0 \text{ if } \text{pH} < \text{pH}_{\text{min}} \text{ or }^{a}W_{v} < {^{a}W_{v}}_{\text{min}}
\]

\[
\mu_{\text{max}}(\text{pH},^{a}W_{v}) = 0 \text{ if } \text{pH} > \text{pH}_{\text{max}} \text{ or }^{a}W_{v} > {^{a}W_{v}}_{\text{max}}
\]

where

\[
\begin{align*}
\mu_{\text{max}}(\text{pH},^{a}W_{v}) &= \mu_{\text{opt}} \cdot \rho(\text{pH}) \cdot \rho(^{a}W_{v}) \text{ if } \text{pH}_{\text{min}} \leq \text{pH} < \text{pH}_{\text{max}} \text{ and } {^{a}W_{v}}_{\text{min}} \leq {^{a}W_{v}} < {^{a}W_{v}}_{\text{max}} \tag{2}
\end{align*}
\]

where

\[
\rho(^{a}W_{v}) = \left(\frac{(^{a}W_{v} - {^{a}W_{v}}_{\text{opt}})^2 \cdot (^{a}W_{v} - {^{a}W_{v}}_{\text{max}})}{({^{a}W_{v}}_{\text{opt}} - {^{a}W_{v}}_{\text{min}}) \cdot ({^{a}W_{v}}_{\text{opt}} - {^{a}W_{v}}_{\text{max}})(^{a}W_{v} - {^{a}W_{v}}_{\text{opt}}) - (^{a}W_{v} - {^{a}W_{v}}_{\text{opt}} - {^{a}W_{v}}_{\text{max}})(^{a}W_{v} - {^{a}W_{v}}_{\text{opt}} + {^{a}W_{v}}_{\text{min}} - 2{^{a}W_{v}})}\right) \tag{3}
\]

and

\[
\rho(\text{pH}) = \left(\frac{(\text{pH} - \text{pH}_{\text{min}})^2 \cdot (\text{pH} - \text{pH}_{\text{max}})}{(\text{pH}_{\text{opt}} - \text{pH}_{\text{min}}) \cdot (\text{pH}_{\text{opt}} - \text{pH}_{\text{max}})(\text{pH} - \text{pH}_{\text{opt}}) - (\text{pH}_{\text{opt}} - \text{pH}_{\text{min}})(\text{pH}_{\text{opt}} + \text{pH}_{\text{min}} - 2\text{pH})}\right) \tag{4}
\]

where \({^{a}W_{v}}_{\text{min}}\) is the \(^{a}W_{v}\) below which growth is no longer observed, \({^{a}W_{v}}_{\text{max}}\) is the \(^{a}W_{v}\) above which no growth occurs and \({^{a}W_{v}}_{\text{opt}}\) is the \(^{a}W_{v}\) at which the maximum growth rate equals its optimal value \(\mu_{\text{opt}}\). Equally, \(\text{pH}_{\text{min}}\) is the pH below which growth is no longer observed, \(\text{pH}_{\text{max}}\) is the pH above which no growth occurs, and \(\text{pH}_{\text{opt}}\) is the pH
at which the maximum growth rate equals its optimal value $\mu_{opt}$.

Untransformed data showed stabilized variance, and for this reason Eqn (2) was used with no further transformation of the 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

The growth of *A. fumigatus* on silage medium in general followed 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 $\mu_{max}$ and $\lambda$ values estimated through Baranyi’s primary model. Regardless of the O$_2$ tension and temperature levels no growth was observed either at 0.8 $a_w$ or at 0.85 $a_w$. Similarly, no growth was observed at pH 3.5, except at 0.94 $a_w$. At the remaining pH levels, in general, there were no 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 $\lambda$ ($P > 0.05$) was found between 25 and 37°C, but a significantly higher $\mu_{max}$ was observed at 37°C ($P < 0.05$). It is clear that there is a great extension of latency ($\lambda$, lamda) at reduced water activities and higher pHs. The increase in the CO$_2$ concentration influenced $\lambda$, leading to a delay in growth initiation, but not on the 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

| $a_w$ | $\mu_{max}$ (mm per day) ± SE | $\lambda$ (day) ± SE | $\mu_{max}$ (mm per day) ± SE | $\lambda$ (day) ± SE | $\mu_{max}$ (mm per day) ± SE | $\lambda$ (day) ± SE | $\mu_{max}$ (mm per day) ± SE | $\lambda$ (day) ± SE |
|-------|-----------------------------|---------------------|-----------------------------|---------------------|-----------------------------|---------------------|-----------------------------|---------------------|
| 0.80 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 |
| 0.80 6 | 0.47 ± 0.03 | 4.87 ± 0.45 | 0.96 ± 0.11 | 5.17 ± 0.21 | 0.52 ± 0.03 | 10.82 ± 0.31 | 0.36 ± 0.04 | 9.49 ± 0.6 |
| 0.80 8 | 0.41 ± 0.02 | 11.19 ± 0.25 | 0.82 ± 0.07 | 33.33 ± 0.2 | 0.40 ± 0.07 | 9.59 ± 0.52 | md | 5.34 ± 0.22 |
| 0.90 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 |
| 0.90 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 |
| 0.90 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 |
| 0.94 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 |
| 0.94 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 |
| 0.96 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 |
| 0.96 8 | 2.01 ± 0.03 | 15.9 ± 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 |
| 0.98 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 |
| 0.98 8 | 5.26 ± 0.09 | 0.29 ± 0.07 | 3.48 ± 0.05 | 0.3 ± 0.1 | 3.39 ± 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 |
| 0.99 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 |
| 0.99 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 |

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

| Parameter | RC031 | RC032 |
|-----------|-------|-------|
| $a_{\text{W}, \text{opt}}$ | 0.85 ± 0.00 | 0.85 ± 0.00 |
| $a_{\text{W}, \text{min}}$ | 0.84 ± 0.01 | 0.84 ± 0.01 |
| $a_{\text{W}, \text{max}}$ | 1.00 ± 0.01 | 1.00 ± 0.01 |
| pH$_{\text{opt}}$ | 2.25 ± 0.14 | 2.25 ± 0.14 |
| pH$_{\text{max}}$ | 11.94 ± 0.61 | 11.94 ± 0.61 |
| pH$_{\text{pH}}$ | 6.58 ± 0.27 | 6.58 ± 0.27 |
| R2 | 0.906 | 0.888 |
| MSE | 0.34 | 0.30 |

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 the growth conditions and strain.

In the pH, temperature, oxygen tension and $a_{\text{W}}$ range studied the optimal conditions for growth for the assayed *A. fumigatus* strains were 0.99–1  $a_{\text{W}}$ and 4.7–6.6 pH with a maximum predicted growth rate of 5.2 mm per day and 5.2–5.7 mm per day at 25°C with and without reduced O$_2$ tension, respectively, and 10.3–10.5 mm per day at 37°C. The estimated $a_{\text{W}, \text{min}}$ varied from 0.84 to 0.86. The estimated pH$_{\text{min}}$ ranged from 2.2 to 2.3 (Table 3). The combined effect of pH and $a_{\text{W}}$ with the different temperature and CO$_2$ treatments is shown in Fig. 1. The most important impact on the growth rate was that of temperature, with much faster growth at 37°C. Moreover, the increase in the growth rate with $a_{\text{W}}$ was 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 a 5% CO$_2$ concentration.
Discussion

The combined effects of CO$_2$, pH, $T$ and $a_W$ on 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 relate to nutrients available in the environment of silage. The use of a rich laboratory medium might have encouraged the growth of $A. fumigatus$, whereas SEM might be closer to the nutrient levels found in a real silage ecosystem. Astoreca et al. (2012), 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, the $a_W$ levels reported in the silages showed values between 0·90 and 0·99. González Pereyra et al. (2011) reported $a_W$ levels

\[ \begin{align*}
0·84 & \quad 0·89 & \quad 0·94 & \quad 0·99 & \quad 3·5 \\
5 & \quad 6·5 & \quad 8 & \quad 0 & \quad 2 & \quad 4 & \quad 6 & \quad 8 & \quad 10 & \quad 12
\end{align*} \]

\[ \text{Figure 1 Multifactorial cardinal model fitted to observed growth rates values of } A. fumigatus \text{ isolates: (a) RC031 5%CO}_2, (b) RC032 5%CO$_2$ (c) RC031 , (d) RC032 to 25°C , (e) RC031, f) RC032 to 37°C, at different $a_W$ and pH levels. [Colour figure can be viewed at wileyonlinelibrary.com] \]
from 0·901 to 0·985 in corn trench silos and silo bags samples in our region. These levels were quite homogeneous in all samples (around 0·98), except for samples from the lower and upper sections of trench silos, where average values were significantly lower. In corn silage samples from Brazil, Keller et al. (2013) reported $a_W$ 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. The 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 the pH decreases to 3·8–5·0. Phase 3: stable phase, better properties are retained for a longer time so preventing the entry of air (Merry et al. 1997). After the silo is opened, the air inlet increases and the pH can rise. According to the mild effect of pH and CO$_2$ on A. fumigatus observed in this study, the preservation strategies may fail to prevent its growth. Moreover, silo compaction is of relevance; when the pH is over 4, the growth of A. fumigatus is optimum. This poses a serious problem in the contamination control of silage, since the basis for its preservation is a pH decrease to 4–5, accompanied by an anaerobic phase. The CO$_2$ levels in silage have not been determined experimentally. However, a fermentation process occurs and it is assumed that a CO$_2$ concentration exists sufficient for micro-organisms to make this an anaerobic process instead of an aerobic one. Normally to simulate microaerophilic in vitro conditions, a standardized 5% CO$_2$ is used. Although initially CO$_2$ inhibits microbial growth, when the silo is open and the oxygen tension increases the A. fumigatus spores present may start to grow, since the $a_W$ levels allow it and a low pH may not help to control it. In relation to the results of our study, the extension of the time to growth ($\lambda$) indicates that a combination of CO$_2$ and low pH would help to inhibit A. fumigatus growth.

Kineti c models for the 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. Marin 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.

The estimated $\mu_{opt}$ values were significantly higher at 37°C than at 25°C; this is not good in the case of an opportunistic pathogen such as A. fumigatus. Temperature variation in silage is usually between 24 and 25°C (Gonzalez Pereyra et al. 2008; Alonso et al. 2009), once the silo is opened. Anyway the rationale for studying the temperature at 37°C refers to the body temperature. Few studies on the influence of environmental factors, specifically temperature, on the growth of A. fumigatus have been reported; 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 the human lung (37°C) and had similar pathogenicity characteristics to clinical strains (Alonso et al. 2016). 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 the 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 was inhibited, however, these data are not relevant for the silage environment, due to the low temperature. Notably there are no reports in the literature regarding the application of mathematical models on the growth of A. fumigatus, Astoreca et al. (2012) modelled the effects of temperature (10–40°C) and $a_W$ (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 $a_W$ and 32–36°C. Growth limits could be estimated by applying these models ($a_W$ 0·79–0·85 and temperature 8·4–10·0°C) for grain corn conservation, but can not 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 the better development of mathematical models applied to A. fumigatus.

In conclusion, this study showed that environmental conditions taking place during silage production, while limiting the growth of most micro-organisms, would not be able to control A. fumigatus. The existing $a_W$ range
allows the growth of *A. fumigatus* and may be even close to its optimum. Moreover, the pH levels found in silage, far from limiting its growth, are also close to its optimum. Finally, a 5% CO₂ environment did not significantly affect its growth. There is a need for a further and controlled acidification of the silage, 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.

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**Conflict of Interest**

The authors of this paper declare that there is no conflict of interest that should be disclosed about this work.

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