The growth and aflatoxin production of *Aspergillus flavus* strains on a cheese model system are influenced by physicochemical factors

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ABSTRACT

Traditional cheeses may be contaminated by aflatoxin-producing *Aspergillus flavus* during the ripening process, which has not been sufficiently taken into account. The objectives of this study were to evaluate the influence of water activity (a_w), pH, and temperature on the lag phases, growth, and aflatoxin production of 3 *A. flavus* strains (CQ7, CQ8, and CG103) on a cheese-based medium. The results showed that the behavior of *A. flavus* strains was influenced by pH, a_w , and temperature conditions. The CQ7 strain showed the maximum growth at pH 5.5, 0.99 a_w , and 25°C, whereas for CQ8 and CQ103 strains, no differences were obtained at pH 5.5 and 6.0. In general, low pH, a_w , and temperature values increased the latency times and decreased the growth rate and colony diameter, although a_w and temperature were the most limiting factors. Maximum aflatoxin production on the cheese-based medium occurred at pH 5.0, 0.95 a_w , and 25 or 30°C, depending on the strain. This study shows the effect of pH, a_w , and temperature factors on growth and aflatoxin production of 3 aflatoxigenic *A. flavus* strains on a cheese-based medium. The findings may help to design control strategies during the cheesemaking process and storage, to prevent and avoid aflatoxin contamination by aflatoxigenic molds.

Key words: aflatoxin, *Aspergillus flavus*, growth, ripened cheese

INTRODUCTION

Mold spores are ubiquitous in nature, with the ability to survive stressful environment conditions and grow on many diverse substrates. Consequently, the contamination of cheese by yeast and molds can occur throughout the production, storage, and distribution chain.

The raw materials, such as milk and coagulant, dairy utensils, and environment can act as potential vectors of microbial contamination (Irlinger et al., 2015). For instance, cardoon flowers (*Cynara cardunculus* L.), a vegetable coagulant typical of the Mediterranean area, constitute an important source of fungal contamination in certain artisanal cheese (Fernández-Salguero et al., 1999; Gómez et al., 2001; Tejada et al., 2008). Moreover, the technological processes applied during cheese manufacture, ripening, and storage [physicochemical (e.g., salting, temperature, water activity, pH, and relative humidity) and biochemical (e.g., smoking, antimicrobial substances, and gas composition of package)] play a major role in the microbial dynamics (McSweeney et al., 2004; Almena-Aliste and Mietton, 2014; Montel et al., 2014).

Overall, molds are an important constituent of the secondary microbiota of cheese and their presence is acceptable, even necessary, due to their involvement in lipid and protein degradation that contribute to the sensorial properties of the final product, playing a key role in texture and flavor development (Molimard and Spinnler, 1996; Fox and McSweeney, 2004; Benkerroum, 2016). In certain cheeses, such as Camembert, Roquefort, or blue cheese, some species of molds, mainly *Penicillium* spp., are intentionally added as ripening starters (Ropars et al., 2012). However, the growth of some mold species can lead to deterioration of the product and production of toxic secondary metabolites called mycotoxins (O'Brien et al., 2004; Hymery et al., 2014). The mold most frequently identified in cheese is *Penicillium* spp., followed by *Mucor* spp. and *Geotrichum candidum* as well as 12 other fungal genera present to a lesser extent (Barrios et al., 1998). On the other hand, the contamination of *Aspergillus* spp. in cheese has been reported by several authors (Barrios et al., 1997; De Santi et al., 2010; Baranyi et al., 2015). Aflatoxins (AF) are highly toxic and carcinogenic mycotoxins produced by *Aspergillus flavus* and the less aggressive and less widely distributed *Aspergillus parasiticus* (Pitt and Hocking, 1999). Aflatoxins B₁ and B₂ are mainly pro-

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duced by *A. flavus*, whereas *A. parasiticus* can produce all 4 AF (B₁, B₂, G₁, and G₂). Aflatoxin B₁ is produced in higher amounts than the other AF. Additionally, it is considered the most toxic natural compound, designated as a group 1 human carcinogen by the IARC (2012). These mycotoxins AFB₁, AFB₂, AFG₁, and AFG₂ have all been detected in cheeses (Taniwaki et al., 2001; Baranyi et al., 2015). In addition, sterigmatocystin, which is structurally related to AF because is produced by the same biochemical pathway from homologous structural gene, has been reported to occur in cheese linked to mold growth (Northolt et al., 1980; Sulyok et al., 2010). However, in general, few studies have documented the occurrence of AF by mold contamination, probably because mycotoxin studies have been more focused on economically important cheeses than on traditional raw milk cheeses, which are more prone to contamination by mycotoxigenic molds.

It is generally accepted that fungal growth and mycotoxin production in food is influenced by multiple factors, such as substrate composition, temperature, a_w , pH, and microbial interactions (Magan and Aldred, 2007). The physicochemical characteristics of cheese are subject to changes during ripening, which influence the mycotoxin production. *Aspergillus flavus* grows and produces mycotoxins at a wide range of temperature and water activity (a_w), although the ideal conditions are 28 to 30°C, at high a_w values (Schmidt-Heydt et al., 2009; Abdel-Hadi et al., 2012). The influence of these physicochemical factors on growth and mycotoxin production by *A. flavus* on cheese-based matrices has not yet been studied.

As a consequence of the intensive labor required, or even impossible processes, to eliminate mycotoxins from food matrix, preventive measures are considered to be the most practical and reasonable way to avoid mycotoxin contamination (Benkerroum, 2016). Therefore, understanding the influence of ecophysiological factors on *A. flavus* growth and AF production using a cheese model would provide valuable information for controlling their occurrence in the final product, thus minimizing the potential hazards associated with AF contamination of cheese due to mold growth.

The objective of this study was to evaluate the influence of a_w , pH, and temperature on the lag phases before growth, growth, and AF production of *A. flavus* on a cheese-based medium.

MATERIALS AND METHODS

Fungal Strains

Three AF-producing *Aspergillus* strains, *A. flavus* CQ7, *A. flavus* CQ8, and *A. flavus* CQ103 (Fungi Cul-

ture Collection of the Agricultural Engineer School, Extremadura University, Badajoz, Spain), previously isolated from *C. cardunculus* L., were used for this work. The spore suspensions were stored at -80°C in glycerol solution (40% wt/vol) before use.

Preparation of Cheese Agar

Cheese agar medium was used to determine the effect of pH, a_w , and temperature on growth and AF production of the 3 *A. flavus* strains. The cheese-enriched medium was prepared as described by Gori et al. (2012), with modifications. Briefly, 200 g/L of soft body matured cheese from raw ewe milk (Ordiales et al., 2013) was added to 400 mL of demineralized water and ground for 5 min using a hand blender. Trisodium citrate dihydrate (12.5 g/L) and NaCl (40 g/L; Sigma-Aldrich, St. Louis, MO) were added, and the mixture was stirred vigorously on a magnetic stirrer and heated until boiling. Subsequently, 20 g/L of Bacto agar (Pronadisa, Madrid, Spain) was added and the medium adjusted to a_w 0.85, 0.90, 0.95, and 0.99 by the addition of 286, 193, 101, and 0 mL of glycerol (Scharlab S.L., Barcelona, Spain) per liter of medium, respectively. Mineralized water was added to complete the volume to 1 L and the culture medium was sterilized by autoclaving at 121°C for 20 min. Lactic acid, sterilized by filtration through a 0.2- μ m filter, was added to adjust the cheese medium to pH 5.0, 5.5, and 6.0, after autoclaving, when the temperature of the medium was ~50°C. Then, the cheese agar was immediately poured into Petri dishes and allowed to solidify at room temperature. The a_w of the cheese agar medium was confirmed by using a Novasina LabMaster- a_w meter (AG, Lachen, Switzerland).

Inoculation of Cheese Agar

For inoculum preparation, malt extract agar (Scharlab, Barcelona, Spain) was inoculated with the strains and incubated at 25°C for 7 d. The spore suspension of each strain was collected by adding 10 mL of sterile aqueous 0.05 mL/100 mL of Tween 80 (Scharlab) to each mold plate, followed by rubbing the surface with a glass rod. The suspension formed, was filtered through 2 layers of cheesecloth. The concentration of each spore suspension was quantified by using a microscope (Olympus CX 400, Tokyo, Japan) and Neubauer chamber. Two microliters of each spore suspension, adjusted to 10⁵ spores/mL with sterile water, was inoculated onto cheese agar plates adjusted to the pre-determined pH and a_w conditions, and incubated at various temperatures (10, 15, 20, 25, and 30°C) to evaluate their influence on the growth of *A. flavus* and AF production.

Experimental Parameters

Initial experiments were performed at 1 fixed temperature (25°C), at 3 pH (5.0, 5.5, and 6.0) commonly encountered in cheese and cheese products. Afterward, the pH was fixed for further experiments to check the effect of a_w (0.85, 0.90, 0.95, and 0.99) on growth and AF production at 25°C for 12 d after the end of the lag phase. Then, with pH and a_w fixed, the experiment was checked at various temperatures (10, 15, 20, 25, and 30°C). For each experiment and condition, 2 biological replicates and 3 technical replicates were performed.

Assessment of Growth and Lag Phase

The diameter of the growing colonies of all replicates under all conditions was measured at 2 perpendicular directions every 48 h during the 12 d after the end of the lag phase. The average of both diameters was recorded as the growth measurement for each strain. Mean colony diameter (mm) of each experiment and condition was plotted against incubation time (d) to establish growth curves for each fungal strain. The colony growth rate [μ (mm/d)] was determined from the slope of the growth curve, whereas the lag phase [λ (d)] was estimated by extrapolating the linear regression equation to the original inoculum size (μ_0 diameter, mm) ($\lambda = \mu_0 \pm b/\mu$, where b is the intercept of the original size).

Preparation of Cheese-Based Agar Samples for AF Extraction

Mycelia were collected at various time points, according to the first visual appearance of fungal colonies under the established pH, a_w , and temperature conditions. Five agar plugs of 4 mm in diameter were removed from each mold colony culture every 48 h during 12 d after the end of the lag phase and placed in 2-mL microcentrifuge tubes. All replicates per treatment were collected and stored at -20°C, before extraction for AF quantification.

AF Extraction

Aflatoxins were extracted from the fungal mycelium by the quick, easy, cheap, effective, rugged, and safe (QuEChERS) procedure (Ferreira et al., 2012), with modifications. For this purpose, 2 agar plugs were placed in an extraction tube containing 200 mg of $MgSO_4$ and 50 mg of NaOAc (both from Fisher Scientific, Madrid, Spain). Then, 1 mL of HPLC-grade acetonitrile (Fisher Scientific) was added and the mixture was shaken vigorously for 2 min, followed by sonication

for 20 min, and then centrifuged at $14,000 \times g$ at 25°C for 1 min. The supernatant was transferred to a clean tube containing 75 mg of $MgSO_4$, 25 mg of primary secondary amine (Sigma-Aldrich, St. Louis, MO), and 25 mg of C18 (Scharlab), and homogenized for 1 min. The mixture was centrifuged ($14,000 \times g$, 25°C, 1 min) and 400 μ L of the supernatant was filtered through a 0.22- μ m pore size filter into vials for quantification.

The AF analysis was carried out using an Agilent 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector (Agilent G1315B) set at 360 nm and using a C18 HPLC column (250 $\times$ 4.6 mm, 5- μ m particle size; Supelco, Bellefonte, PA). The mobile phase used for the separation contained HPLC grade water (solvent A) and HPLC grade acetonitrile (solvent B), run in a gradient mode set from 15% B at initial to 100% B at 30 min. A 3-min period was necessary for column equilibration. All solvents used were purchased from Thermo Fisher Scientific (Runcorn, UK). The injection volume was 25 μ L and the flow rate was 1 mL/min for all the experiments. Calibrations were carried out for each AF using standards purchased from Sigma-Aldrich.

Statistical Analysis

The physicochemical and growth parameters and toxin production were compared during the sampling time using a 1-way ANOVA. The means were separated by Tukey's honest significant difference test ($P < 0.05$) using SPSS for Windows 21.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Effect of pH, a_w , and Temperature on the Growth of *A. flavus*

The effects of the evaluated physicochemical parameters on the colony diameter and growth parameters of the 3 AF-producing *A. flavus* strains isolated from *C. cardunculus* L. flowers, are presented in Figure 1 and Table 1, respectively. At 0.99 a_w at 25°C, the pH affected colony growth and was evidently strain dependent. Among the strains, CQ7 presented better ability to grow at lower pH (5.0 and 5.5), displaying the lowest colony diameter and growth rate, whereas it had the longest lag phase ($P < 0.05$) at pH 6.0. In contrast, *A. flavus* CQ8 and CQ103 showed similar behavior at the 3 pH tested, with significantly less growth at pH 5.0 ($P < 0.05$; Figure 1). For both CQ8 and CG103, although the lag phase was not affected by the pH, a significant reduction in growth rate and colony diameter was observed at pH 5.0 (Table 1). Kosegarten et al. (2017) studied the influence of various factors on the growth

of *A. flavus*, observing that the optimum growth rate occurred between pH 3.5 to 6.0, although they showed that the growth of *A. flavus* was less affected by pH than other environmental factors. These studies are in line with the results obtained in the present work.

Regarding the influence of a_w on growth at pH 5.0 at 25°C, the 3 *A. flavus* strains were not able to grow at 0.85 a_w . Pitt and Miscamble (1995) determined that a minimum 0.80 to 0.83 a_w was necessary to support the growth of *A. flavus*. However, other authors shown a lack of or marginal growth at 0.90 a_w at 25°C (Medina et al., 2015; Lahouar et al., 2016). As shown in Figure 1, CQ7 did not grow at 0.90 a_w , whereas CQ8 and CQ103 showed the minimum mean values of growth rate and colony diameter (Table 1). At 0.95 or 0.99 a_w , the growth was more favorable for the 3 strains, although they presented significant differences at the optimal a_w (Figure 1; Table 1). Among the strains, CQ7 showed the best ability to grow at both a_w , with a higher colony diameter and growth rate and a shorter lag phase under both conditions. Although CQ8 presented a lower growth capacity than CQ7, both exhibited similar growth tendency, with the highest colony diameter, growth rate, and lowest lag phase at 0.99 a_w . In contrast, CQ103 showed the maximum colony diameter and growth rate at 0.95 a_w ($P < 0.05$; Figure 1; Table 1). It is well known that *A. flavus* is highly sensitive at ~ 0.90 a_w at 25°C and exhibits optimum growth between 0.95 to 0.99 a_w . Overall, our results are in line with those described by other authors for this species on other media or in food matrix models. Lahouar et al. (2016) observed that a minimum 0.91 a_w was required for mycelial growth at 25°C, whereas Mousa et al. (2011), using a paddy grains model, showed that a minimum 0.86 a_w allowed the growth of *A. flavus*. Conversely, Abdel-Hadi et al. (2012) observed that the optimum growth of *A. flavus* occurred at 0.99 a_w at 30 to 35°C during storage. More recently, Peromingo et al. (2016), using a meat model system, and Gallo et al. (2016), using an almond medium, showed that the most favorable conditions were at ~ 0.95 a_w at 25 and 28°C, respectively.

The effect of temperature was determined at values ranging from 10 to 30°C, at 0.95 a_w , pH 5.0. The highest colony diameter and best growth parameters were observed at 25°C for CQ7, whereas CQ8 and CQ103 reached their maximum growth at 30°C (Figure 1; Table 1). Temperatures below 20°C significantly influenced the colony diameter and growth rate of the 3 strains. At 10°C, mold growth was not detected for any strains and at 15 and 20°C, a noticeable effect was observed, with colony diameter below 30 mm at 12 d of growth (Figure 1; Table 1). These results agree with those obtained by Gallo et al. (2016), who observed that the ideal

temperature for the growth of *A. flavus* on an almond medium at 0.96 a_w was at 28°C when compared with 20 and 37°C. Likewise, other authors have reported that the optimum growth conditions for this species, on various substrates, occur at 25 to 30°C, at ~ 0.95 a_w (Lahouar et al., 2016; Peromingo et al., 2016; Kosegarten et al., 2017). In contrast, temperatures below 20°C have widely demonstrated to considerably reduce *A. flavus* growth, with a growth limit $\sim 10^\circ\text{C}$ (Sautour et al., 2002; Abdel-Hadi et al., 2012; Astoreca et al., 2012; Medina et al., 2015; Peromingo et al., 2016).

The behavior of the 3 *A. flavus* strains studied under the various pH, a_w , and temperature conditions, was confirmed by the growth parameters including growth rate (μ), lag phase (λ), and maximum growth ($y_{\max}$; Table 1). The CQ7 strain showed the maximum growth at pH 5.5, 0.99 a_w , 25°C, whereas for the other 2 strains, no differences were observed at pH 5.5 and 6.0. Figure 2 shows an example of the effect of the various conditions studied on the colony growth of CQ8. In general, low pH, a_w , and temperature values increased the latency times and had a greater effect on the growth rate and colony diameter, although a_w and temperature were the most limiting factors (Figure 1; Table 1), corroborating similar, previous studies (Schmidt-Heydt et al., 2008; Kosegarten et al., 2017). The slight divergences found among the literature and between our study and other works concerning the specific critical values of pH, a_w , and temperature, depend on intrinsic and extrinsic factors, such as the strains studied, nutrient composition of the medium, and methods used.

Effect of pH, a_w , and Temperature on AF Production by *A. flavus*

Table 2 shows the effect of pH variation on AF production by the *A. flavus* strains on the cheese model medium. At the 3 pH tested, at 0.99 a_w and 25°C, only AFB₁ was found, with values ranged from no detection to 7.46 $\mu\text{g}/\text{cm}^2$. In general, the production of AFB₁ was significantly affected by the pH and growth time ($P < 0.05$). The 3 strains displayed dissimilar AF production patterns during the growth period. The CQ7 showed a relatively lower capacity to produce AFB₁, with only slight production detected at pH 5.0 at 10 d. The CQ7 produced the maximum 2.73 $\mu\text{g}/\text{cm}^2$ of AFB₁ at pH 5.0 at 12 d, whereas production occurred earlier for the other 2 strains (d 4). The CQ103 started to produce AFB₁ at pH 5.5 at 4 d and at pH 5.0 at d 6, with similar mean values, ranging from 0.81 to 1.88 $\mu\text{g}/\text{cm}^2$, at both pH at d 6, whereas no production was detected at pH 6.0. The most versatile strain was CQ8, which produced AFB₁ under all pH conditions after d 4, showing a significantly greater amount of the toxin at higher pH

Figure 1. Effect of water activity (0.90, 0.95, and 0.99), pH (5.0, 5.5, and 6.0), and temperature (15, 20, 25, and 30°C) on radial growth (mm) of 3 *Aspergillus flavus* strains (CQ7, CQ8, and CQ103) on a cheese-based medium for 12 d after the end of the lag phase.

Table 1. Effect of pH, water activity (a_w), and temperature (T) on growth parameters [growth rates (μ), lag phase (λ), and maximum growth (y_{max})] of 3 *Aspergillus flavus* strains on a cheese-based medium

Factor			Growth parameter								
			<i>Aspergillus flavus</i> CQ7			<i>Aspergillus flavus</i> CQ8			<i>Aspergillus flavus</i> CQ103		
T (°C)	a_w	pH	μ (mm/d)	λ (d)	y_{max} (mm)	μ (mm/d)	λ (d)	y_{max} (mm)	μ (mm/d)	λ (d)	y_{max} (mm)
25	0.99	6.0	4.56 ^{cd,B}	2.44 ^{d,A}	55.50 ^{b,B}	6.66 ^{a,A}	2.48 ^{cd,A}	79.83 ^{b,A}	6.12 ^{b,A}	1.96 ^{b,B}	73.50 ^{b,A}
25	0.99	5.5	7.21 ^{a,A}	0.94 ^{e,B}	101.20 ^{a,A}	6.54 ^{a,B}	2.31 ^{d,A}	76.33 ^{b,B}	5.96 ^{b,B}	2.02 ^{b,A}	72.00 ^{b,B}
25	0.99	5.0	6.15 ^{b,A}	0.55 ^{e,C}	100.00 ^{a,A}	4.87 ^{b,B}	2.25 ^{d,A}	65.17 ^{c,B}	3.45 ^{d,C}	1.62 ^{b,B}	50.00 ^{c,C}
25	0.95	5.0	5.50 ^{bc,A}	5.00 ^{c,B}	79.67 ^{a,A}	2.28 ^{d,C}	6.91 ^{b,A}	34.50 ^{e,C}	4.82 ^{c,B}	4.27 ^{b,C}	69.50 ^{b,B}
25	0.90	5.0	—	—	—	2.16 ^D	6.28 ^b	32.50 ^e	2.26 ^e	4.34 ^b	32.17 ^e
30	0.95	5.0	4.90 ^{cd,B}	4.37 ^{c,A}	64.50 ^{b,B}	6.59 ^{a,A}	1.64 ^{d,A}	86.00 ^{a,A}	6.76 ^{a,A}	1.25 ^{b,A}	86.00 ^{a,A}
20	0.95	5.0	1.51 ^{f,B}	7.39 ^{b,A}	20.00 ^{e,B}	1.90 ^{d,AB}	2.09 ^{d,B}	31.33 ^{ef,A}	2.06 ^{e,A}	3.90 ^{b,AB}	29.00 ^{e,A}
15	0.95	5.0	1.35 ^{f,A}	12.52 ^{a,C}	30.50 ^{d,A}	0.90 ^{e,B}	28.92 ^{a,A}	27.00 ^{f,A}	1.27 ^{f,AB}	17.87 ^{a,B}	29.25 ^{e,A}

^{a-f}Values with different lowercase superscripts are significantly different ($P < 0.05$) between conditions within the same parameter and strain.

^{A-D}Values with different uppercase superscripts are significantly different ($P < 0.05$) between parameters within the same condition.

(5.5 and 6.0 vs. 5.0) throughout the growth time, except at d 12, where there were not statistical differences between pH. It is well known that pH can influence the regulation of the pathways involved in mycotoxin biosynthesis by various mold species including *Aspergillus* spp. (Keller et al., 1997; Schmidt-Heydt et al., 2008). In general, the production of mycotoxins is usually higher at low pH. Keller et al. (1997) reported a higher AF production by *A. parasiticus* at pH 4.0 to 6.0 than at pH 7.0 and 8.0, with a noticeable reduction in the accumulation of AF and a delay in the expression level of the homologous sterigmatocystin/AF structural gene with increasing pH. However, the optimal pH for AF production may vary depending on the strain and medium composition. For example, in 2 *A. flavus* strains, Patel et al. (2014) showed increased AFB₁ production at pH ~6.5 at 27°C compared with acid pH, with slight differences between strains. In our work, although the pH tested were in close proximity, discrete patterns in AFB₁ production were evident (Table 2).

A prominent effect of a_w variation was observed at pH 5.0 at 25°C on AF production in all the strains tested (Figure 3; Table 2). The 2 extreme values of 0.90 and 0.99 a_w were stress conditions for AF production by the 3 strains. At 0.90 a_w , the strains CQ7 and CQ103 were not able to produce AF, whereas CQ8 showed slight accumulations of 0.66, 1.91, 0.99, and 1.09 $\mu\text{g}/\text{cm}^2$ at 6, 8, 10, and 12 d. At 0.99 a_w , a certain AFB₁ production was accumulated by the 3 strains, in the range of 0.87 to 3.42 $\mu\text{g}/\text{cm}^2$. During growth at 0.99 a_w , CQ8 began to accumulate AF from d 4, which was earlier than CQ103 and CQ7, which began at d 6 and 10, respectively. The maximum AF accumulation was evident at 0.95 a_w . At this a_w , all the strains produced AFB₁ and AFB₂ but at different levels during the growth period (Figure 3). Overall, similar amounts of

AFB₁ were produced by the 3 strains, with ~40 $\mu\text{g}/\text{cm}^2$ after d 6 of growth, except CQ7, which reached ~70 $\mu\text{g}/\text{cm}^2$ at d 4 (Figure 3). Regarding AFB₂ production, discrete differences in the amounts produced were observed between strains. The CQ8 and CQ103 strains showed a limited capacity to produce this AF, with levels below 5 $\mu\text{g}/\text{cm}^2$ detected, whereas, CQ7 showed a similar ability to produce both AF. Production of these 2 AF at the same ratio is unusual in *A. flavus* because, generally, this species is considered to produce mainly AFB₁ (Gallo et al., 2016; Lahouar et al., 2016). The AF production was strongly affected by the a_w , with optimum production at 0.95 a_w on the cheese-based medium. These results, in general, concur with those reported in the literature for *A. flavus*. Astoreca et al. (2014) concluded that AF production may occur at 0.86 to 0.98 a_w . Although ~0.83 a_w could be considered the lower limit for production, maximum production is reported to occur from 0.95 to 0.99 a_w at ~25 to 30°C (Giorni et al., 2011; Astoreca et al., 2014; Gallo et al., 2016; Peromingo et al., 2016). However, in the current study, a significant decrease in AF production was detected between 0.95 and 0.99 a_w that may be due to the important influence of the medium characteristics and also the strains studied in the determination of optimal conditions (Klich, 2007).

The influence of temperature variation on AF production was also evaluated in the range 15 to 30°C (Figure 3). As observed for a_w , a noticeable effect of temperature variation was observed among the 3 strains assayed. These 2 abiotic factors have shown to have a more pronounced effect than pH on the regulation of mycotoxin biosynthesis genes in *Aspergillus* spp. (Schmidt-Heydt et al., 2008). In this study, *A. flavus* strains did not produce AF at 15°C, whereas at 20°C, a relatively low amount was detected and the accumula-

Figure 2. Colony growth of *Aspergillus flavus* CQ8 on a cheese-based medium under various conditions. (A) pH: 6.0, 5.5, and 5.0 at 8 d after the end of the lag phase; (B) 0.99, 0.95, and 0.90 water activity (a_w) at 8 d after the end of the lag phase; (C) incubation temperature: 15, 20, 25, and 30°C at 8 d after the end of the lag phase. Color version available online.

Table 2. Effect of pH (5.0, 5.5, and 6.0) at 0.99 water activity and 25°C on aflatoxin production ($\mu\text{g}/\text{cm}^2$) by 3 *Aspergillus flavus* strains for 12 d after the end of the lag phase

Strain	pH	Days					
		2	4	6	8	10	12
CQ7	6.0	<0.04 ^{B†}	<0.04 ^B	<0.04 ^B	<0.04 ^B	<0.04 ^{b,B}	0.66 ± 0.02 ^{b,A}
	5.5	<0.04 ^C	<0.04 ^C	<0.04 ^C	<0.04 ^C	<0.04 ^{b,B}	0.81 ± 0.05 ^{b,A}
	5.0	<0.04 ^C	<0.04 ^C	<0.04 ^C	<0.04 ^C	0.87 ± 0.11 ^{a,B}	2.73 ± 0.50 ^{a,A}
CQ8	6.0	<0.04 ^D	7.46 ± 0.18 ^{a,A}	2.73 ± 0.01 ^{a,C}	3.91 ± 0.081 ^{a,B}	2.85 ± 0.09 ^{a,C}	3.02 ± 0.13 ^{a,C}
	5.5	<0.04 ^E	4.96 ± 0.13 ^{b,A}	2.58 ± 0.08 ^{a,D}	4.08 ± 0.10 ^{a,B}	2.96 ± 0.15 ^{a,C}	2.63 ± 0.22 ^{a,CD}
	5.0	<0.04 ^D	1.50 ± 0.10 ^{c,C}	2.06 ± 0.39 ^{b,BC}	3.42 ± 0.69 ^{b,A}	1.97 ± 0.03 ^{b,BC}	2.67 ± 0.11 ^{a,AB}
CQ103	6.0	<0.04	<0.04 ^b	<0.04 ^c	<0.04 ^c	<0.04 ^b	<0.04 ^b
	5.5	<0.04 ^E	6.88 ± 0.17 ^{a,A}	0.91 ± 0.08 ^{b,D}	1.29 ± 0.04 ^{b,C}	1.55 ± 0.09 ^{a,B}	1.36 ± 0.09 ^{a,BC}
	5.0	<0.04 ^C	<0.04 ^{b,C}	1.33 ± 0.14 ^{a,B}	1.88 ± 0.14 ^{a,A}	1.47 ± 0.04 ^{a,B}	1.48 ± 0.05 ^{a,B}

^{a-c}Values with different lowercase superscripts are significantly different ($P < 0.05$) between conditions with equal day and strain.

^{A-E}Values with different uppercase superscripts are significantly different ($P < 0.05$) between days for one condition.

†For 1-way ANOVA, the samples under the limit of detection were set at 0.

Figure 3. Effect of temperature variation (15, 20, 25, and 30°C) on aflatoxin production by *Aspergillus flavus* strains [(A) aflatoxin B₁, (B) aflatoxin B₂] on a cheese-based medium at 0.95 water activity and pH 5 for 12 d after the end of the lag phase. Values with different letters (a–d) are significantly different ($P < 0.05$) between conditions with equal day and strain. Values with different numbers (1–5) are significantly different ($P < 0.05$) between days for one condition.

tion was delayed compared with the values obtained at 25 and 30°C. At these higher temperatures (25 and 30°C), a significant increase in AFB₁ and AFB₂ was observed for the 3 strains, with production after 2 d of growth, in all instances. The strains CQ8 and CQ103 showed a similar pattern of AF accumulation with the temperature variation. Both strains reached optimum production at 30°C, where at 12 d of growth, ~120 and 160 µg/cm² of AFB₁ was recorded for CQ8 and CQ103, respectively. On the contrary, CQ7 presented a significantly higher AFB₁ and AFB₂ production at 25°C, with

~40 µg/cm² for both AF at 12 d of growth. Previous studies on this species have reported that optimum AF production at 25 to 30°C, regardless of the medium used, whereas an important decrease in production is usually found below 20°C or above 35°C (Giorni et al., 2007; O'Brian et al., 2007; Astoreca et al., 2014; Medina et al., 2015; Gallo et al., 2016; Peromingo et al., 2016).

To the best of the authors' knowledge, this is the first published work to document the effect of various abiotic factors on *A. flavus* in a cheese model system. Overall, the maximum AF production on the cheese-

based medium was obtained at pH 5.0, 0.95 a_w , at 25 or 30°C, depending on the strain. These conditions were, in general, slightly lower than those obtained for optimal growth. Moreover, a minor variation in the conditions implied an important decrease in AF accumulation, despite considerable growth (Figures 1 and 3). Therefore, AF production occurred within a narrow range when compared with the mold growth. Similar findings were found by Abdel-Hadi et al. (2012) and Gallo et al. (2016) on synthetic yeast extract-sucrose medium and an almond system model, respectively. The production of AF by *A. flavus* in response to abiotic factors has been addressed in previous works but the optimal conditions reported for growth and AF production are not always consistent (Schmidt-Heydt et al., 2009; Abdel-Hadi et al., 2012). Abiotic conditions that are slightly lower than those considered optimal for growth have been shown to lead to high amounts of AF, whereas more extreme stress conditions result in inhibitory production (Schmidt-Heydt et al., 2009; Abdel-Hadi et al., 2012). In addition, the biosynthesis of AF can be switched on when the growth is high and the medium is depleted in nutrients (Schmidt-Heydt et al., 2008). The influence of abiotic factors on regulating the biosynthesis of genes involved in secondary metabolite production has not yet been correlated with the primary metabolism for growth (Schmidt-Heydt et al., 2008; Magan and Medina, 2016). Understanding the ecological conditions for growth and AF production of *A. flavus* in a cheese model is decisive to adopting preventive actions in the dairy industry aimed at eliminating AF occurrence.

CONCLUSIONS

The current study showed the effect of environmental factors, including pH, a_w , and temperature, on the growth and aflatoxin production of 3 aflatoxigenic *A. flavus* strains on a cheese-based medium. The results described provide valuable knowledge about the optimal and limiting conditions for growth and AF production that may help researchers design and implement effective control strategies to avoid contamination by aflatoxigenic molds during the manufacture and storage of cheese.

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