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RESEARCH ARTICLE

# Comparison of the growth and aflatoxin production of *Aspergillus parasiticus* on in-shell, shelled and split almonds depending on water activity and temperature

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**Abstract**

*Aspergillus parasiticus* is a fungus that can infect almonds and produce carcinogenic aflatoxins (AF). The objective of this study was to determine the growth and total AF production of *A. parasiticus* on three types of almond kernels (in-shell, shelled and split) under different combinations of temperature (20, 27 and 35 °C), water activity (0.65, 0.75, 0.80, 0.85, 0.90, 0.95, 0.99 $a_w$) and incubation period (10, 20 and 30 days). The in-shell kernels supported the least amount of growth and AF production. The fungus grew moderately at 0.90 and 0.95 $a_w$ and produced moderate (<50 μg/kg) amount of total AF at 0.95 $a_w$ on in-shell almonds. On shelled kernels, growth was also limited to 0.90 and 0.95 $a_w$. Aflatoxin production reached high levels at 0.95 $a_w$ at all three temperatures on shelled almonds (324.3, 325.4, and 139.8 μg/kg at 20, 27 and 35 °C, respectively). The fungus grew rapidly and produced high levels of AF (>300 μg/kg) on split kernels over a wide range of conditions (0.80 to 0.95 $a_w$ and 20 to 35 °C). Contour plots revealed that the optimum conditions for AF production on split kernels were at 0.90-0.95 $a_w$ and 20-27 °C. Aflatoxin production also depended on incubation time. By day 30, AF production on split kernels exceeded 300 μg/kg at 0.80 $a_w$ at all temperatures. There was no fungal growth and AF production at 0.65 and 0.75 $a_w$. These findings indicate that maintaining low water activity (<0.75 $a_w$) during transport and storage can reduce the risk of infestation and aflatoxin production by *A. parasiticus* on inshell, shelled and split almond kernels.

**Keywords**

aflatoxins – almonds – *Aspergillus parasiticus*

## 1 Introduction

Almonds (*Prunus amygdalus*) are edible, nutrient rich tree nuts cultivated in Mediterranean climates worldwide. The California almond industry is the largest in the world, producing about 80% of the total global almond production both for domestic consumption and international trade, with a market value of 5.47 billion dollars (Rane *et al.*, 2023).

Aflatoxins are hepatotoxic and carcinogenic substances produced by *Aspergillus* fungal species, which are ubiquitous in the environment. Aflatoxins are divided into four types ($AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$) of which $AFB_1$ is the most toxic. The International Agency for Research on Cancer (IARC) categorised all four types of aflatoxins as highly carcinogenic (group 1) (IARC, 2002). Because of the detrimental health effects of consuming aflatoxin-contaminated food, current regu-

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lations set limit on the levels of aflatoxins allowed in food. The European Union set the limits at 8.0 μg/kg for $AFB_1$ and 10.0 μg/kg for total aflatoxins in ready-to eat almonds (EC, 2023). The United States Food and Drug Administration (FDA) set the legal limit for total aflatoxin at 20 μg/kg (National Grain and Feed Association, 2024). These standards have a significant impact on the domestic and global trade of almonds and can result in serious economic losses to the almond industry (Rane *et al.*, 2023). Recent surveys of ready-to-eat nuts in different countries indicated that $AFB_1$ and total AF levels exceeded the permissible legal limit, including in Italy (Vita *et al.*, 2022), Algeria (Belasli *et al.*, 2023), and India (Anshida*et al.*, 2023).

*Aspergillus flavus* and *Aspergillus parasiticus* are the main sources of AF contamination in almonds (Rodrigues *et al.*, 2012). In field samples, *A. flavus* is the dominant *Aspergillus* species naturally infecting almonds (Donner *et al.*, 2015), and most studies involving aflatoxins on almonds to date have focused on *A. flavus* (Gallo *et al.*, 2016; Giorni *et al.*, 2011; Gradziel *et al.*, 1994; Rane *et al.*, 2023; Rodrigues *et al.*, 2012); fewer studies have been devoted to determining growth conditions and AF contamination of almonds by *A. parasiticus*. Although *A. parasiticus* is thought to infect almonds less frequently, this mould is known to be a consistent producer of $AFB_1$. Therefore, even a mild level of *A. parasiticus* infestation could lead to high levels of $AFB_1$ contamination, which would result in major crop losses and rejection from global markets. Indeed, it was estimated that in California, *A. parasiticus* was responsible for >50% of nut batches exceeding the regulatory limit of 20 μg/kg set up by the FDA (Garcia-Lopez *et al.*, 2018). Therefore, determining the environmental conditions that favour the growth of *A. parasiticus* on almonds is essential for reducing economic losses in the almond industry.

Fungal contamination of almonds can occur in the field before harvest, during harvest, or during transport and storage (Turner *et al.*, 2005). Once crops are contaminated, it is difficult to eliminate aflatoxins from the food with current food processing techniques, because aflatoxins are extremely stable molecules even at high temperature (Ebrahimi *et al.*, 2022). Consequently, establishing postharvest conditions that reduce the risk of infestation with aflatoxin-producing moulds is critical to avoid crop spoilage.

Various factors before and after harvest, particularly the temperature and humidity of the environment, affect the occurrence of moulds and aflatoxins on almonds (Gallo *et al.*, 2016). Furthermore, in California, infestation by the Lepidopteran navel orangeworm (NOW) has been shown to be strongly associated with fungal invasion and AF contamination of almonds (Moral *et al.*, 2022). Also, even under similar environmental conditions, the degree of fungal spoilage and AF accumulation could be markedly different depending on the type of substrate (seed type). However, studies that compare the growth and AF production of *A. parasiticus* on different types of almond kernels are lacking. At the same time, this information would be critically important to almond producers, because different types of kernels may require different storage or shipping conditions to avoid losses due to AF generation from and growth of moulds such as *A. parasiticus*.

The goal of this study was therefore to reduce the present knowledge gaps. The objectives were to (1) study the differences in growth and total AF production of *A. parasiticus* on three types of almond kernels, (2) determine the optimum temperatures and water activities for growth and total AF production of *A. parasiticus* on different almond kernels, and (3) investigate the role of the length of incubation period on growth and total AF production of *A. parasiticus* on almond kernels. These objectives were achieved by incubating *A. parasiticus* on in-shell, shelled and split almond kernels under various environmental conditions, which were carefully selected to represent a comprehensive range of conditions that might occur post-harvest. The findings of this study will be useful for almond producers in optimising storage and transport conditions for the different types of almond kernels to reduce the risk of AF contamination by *A. parasiticus*.

## 2 Materials and methods

### *Almond kernels*

Commercial Californian almonds (cultivar Nonpareil) were acquired from the Almond Board of California in 2023 and were stored in plastic bags at 4 °C. Three types of kernels were used in this study, specifically: (1) whole, in-shell almonds; (2) whole, shelled (i.e. shell removed) almonds, and (3) split almond kernels. Before the study, all almond samples had been previously pasteurised using commercial propylene oxide (PPO) fumigation (Almond Board of California, 2008).

### *Fungal culture*

*A. parasiticus* (NRRL 465), which can produce all four types of aflatoxins, was acquired from the United States Department of Agriculture (USDA). The strain was cul-

tured in sterile Petri dishes (100 mm × 15 mm) on Potato Dextrose Agar (Thermo Fisher Scientific, Waltham, MA, USA) at 27 °C for 5 days. After 5 days of incubation, spores were suspended in 5 ml of 0.05 % Tween 80 solution. The spore suspension was adjusted to 0.25 OD at 540 nm using a spectrophotometer (Spectronic 20 Genesys, Thermo Fisher Scientific). The spore count was visually confirmed with a hemacytometer (INCYTO, Cheonan-si, Korea). The suspension had a spore concentration of approximately $10^5$ spores/ml.

### *Experimental setup*

The experiments were conducted in 60 × 15 mm sterile Petri dishes (Corning, NY, USA). Each sample consisted of either four pieces of in-shell almonds (8 g/sample), or eight shelled almonds (8 g/sample), or 10 split almond kernels (4 g/sample). The kernels were placed in a single layer. The initial water activity levels of the kernels were as follows: 0.25 $a_w$ (in-shell), 0. 26 $a_w$ (shelled) and 0.23 $a_w$ (split). The water activity of each sample was adjusted to the desired level by adding autoclaved, deionized (DI) water into the Petri dishes using sterile pipette tips as follows. For the inshell and shelled kernels, 100, 300, 500, 800, 1000, 2000, 3000 µl of water was added to achieve 0.65, 0.75, 0.80, 0.85, 0.90, 0.95 and 0.99 $a_w$, respectively. For the split kernels, 50, 100, 150, 300, 600, 900, 1500 µl of water was added to achieve 0.65, 0.75, 0.80, 0.85, 0.90, 0.95 and 0.99 $a_w$, respectively. The dishes were shaken to make sure that the water was equally distributed among the almonds. The equilibrium time to reach the desired water activity after inoculation was 48 h. The water activity of the samples was maintained by incubating the sealed Petri dishes (Petri-Seal Adhesive Sealing Film, CBS Scientific, San Diego, CA, USA) in closed Mason jars, and placing wet paper towels inside the jars. The water activities were monitored throughout the experiment every 5 days using the water activity meter HygroPalm23$A_w$ instrument (Rotronic, Bassersdorf, Switzerland).

After adjusting the water activities, the samples were point inoculated with 15 µl of spore suspension (approximately $10^5$ spores/g of almond) using sterile pipette tips (Corning). The split kernels were inoculated on the flesh surface. Samples were incubated for 10,20 or 30 days at a given combination of water activity (0.65, 0.75, 0.80, 0.85, 0.90, 0.95 or 0.99 $a_w$) and temperature (20, 27 or 35 °C) in triplicates. In addition, all samples with 0.65 $a_w$ were monitored for fungal growth for a 6-month observation period. All samples were visually inspected for fungal growth with a 2× magnifying glass every 5 days. The number of infected almonds in each dish was recorded. For triplicates of the same experimental condition, the average of the number of infected almonds was calculated and reported.

### *Aflatoxin extraction*

Aflatoxin was extracted from triplicate samples at 10, 20 and 30 days of incubation. The contents of each triplicate samples were combined, and the manufacturer's instructions were followed for the extraction of aflatoxins (VICAM, 2020). For the in-shell and shelled almonds, 24 g of the sample was added to a blender with 4.8 g salt (NaCl) and 120 ml of methanol:water (60:40) solution. For the split samples, 12 g of sample was added with 2.4 g of salt and 60 ml of methanol: water (60:40) solution. The mixture was blended at high speed for 60 s. The contents of the blender were then filtered into 50 ml sterile plastic centrifuge tubes using a plastic funnel and PF Filter Paper (Thermo Fisher Scientific). Next, 10 ml of the filtered extract was mixed with 10 ml of DI water. The diluted extract was filtered again into a clean tube with a sterile 25 mm syringe filter (Thermo Fisher Scientific).

### *Aflatoxin purification*

The filtrate was then purified using immunoaffinity columns (Vicam, Milford, MA, USA) as follows. First, 10 ml of the filtered diluted extract was loaded onto the column and allowed to pass completely. Next, 10 ml of DI water was passed through the column, and this step was repeated twice. Finally, the AF was eluted with 1.0 ml of HPLC-grade methanol. The flow rate of the pump was 1 drop/second. The eluted samples were collected into SureSTART™ 2.0 ml glass vials (Thermo Fisher Scientific) and stored in the freezer until further analysis.

### *High Performance Liquid Chromatography analysis*

Aflatoxin analysis was carried out using Thermo Scientific Ultimate 3000 HPLC equipped with fluorescence detector. Isocratic mobile phase of 50% water, 40% methanol and 10% acetonitrile (HPLC grade solvents, Thermo Fisher Scientific) with a flow rate of 1 ml/min was used to elute $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ through a reverse phase column C18, 4.6 mm x 250 mm stationary phase. The chromatogram was recorded at 365 nm excitation and 455 nm fluorescence emission. Four separate standard calibration curves for each type of aflatoxin were made using standard solutions (Sigma-Aldrich, Milwaukee, WI, USA). For example, for $AFB_1$, eight standard samples in the concentration range of 2.5 to 1000 µg/kg of $AFB_1$ were used to draw the calibration curve.

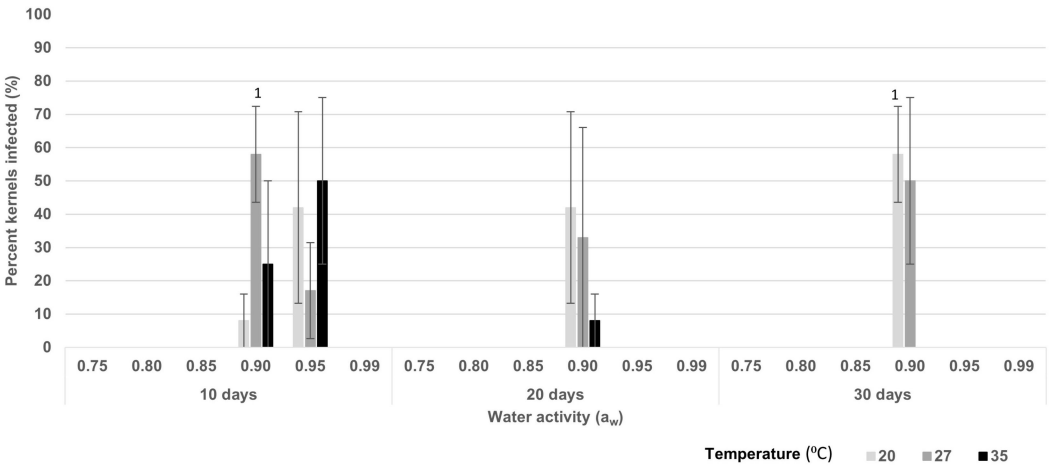


FIGURE 1 Growth of *Aspergillus parasiticus* on in-shell almond kernels at various combinations of water activity and temperature at 10, 20 and 30 days of incubation. Error bars represent standard deviation. Numbers denote significantly different values within the dataset based on Tukey's multiple comparisons test.

All the four calibration curves for the four aflatoxins obtained proved linear with $r^2 > 0.996$. The retention time of $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ were approximately 8.20, 7.12, 5.98, 5.27 min, respectively, and the detection limit was 1.5 μg/kg for $AFB_1$ and $AFG_1$ while the detection limit was 1 μg/kg for $AFB_2$ and $AFG_2$ under the conditions described above. The coefficient of variation (%CV) and the recoveries of samples spiked with 10 μg/kg of $AFB_1$ were 0.6% and 78.9%, respectively, which is consistent with the guidelines of recovery rate 70-110% of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for analysis of aflatoxin (Benford *et al.*, 2001; Kim *et al.*, 2017). Total aflatoxins were obtained by adding the four types of aflatoxins present in the samples.

### *Statistical analysis*

The proportion of infected kernels in each Petri dish was calculated by dividing the number of infected kernels by the total number of kernels in the Petri dish. The proportions were expressed as percentages. The effects of temperature, water activity and their interaction on fungal growth on the split almond kernels on days 10, 20 and 30 were statistically analysed by analysis of variance (ANOVA**).** Tukey's multiple comparison test was used to compare the differences in growth and aflatoxin production under different combinations of temperature and water activity. The means, standard deviations and Tukey's test of total aflatoxin production on split almond kernels was calculated based on aflatoxin measurements taken at 10, 20 and 30 days of observation. A *P*-value <0.05 was considered statistically significant. Contour plots were prepared using STATA IC 15 software (College Station, TX, USA).

## 3 Results

### *Fungal growth and total aflatoxin production on in-shell and shelled almond kernels*

Overall, *A. parasiticus* did not grow well on in-shell (Figure 1) and shelled (Figure 2) kernels and initial growth declined over time. The fungus showed some growth at 0.90 and 0.95 $a_w$ on in-shell and shelled kernels in the first 10 days at all three temperatures. At 20 days, growth was mostly limited to 0.90 $a_w$ at all temperatures for both in-shell and shelled almonds There was also some growth at 27 °C and 0.95 $a_w$ on the shelled kernels at 20 days. By day 30, there was only growth at 0.90 $a_w$ and 20 and 27 °C for in-shell and 20 °C for shelled almonds.

Even though there was fungal growth at 0.9 $a_w$ on in-shell and shelled almonds, there was no AF production at any of the study temperatures at this water activity. Total aflatoxin production by *A. parasiticus* on in-shell and shelled almonds was limited to 0.95 $a_w$ at all the three temperatures. For in-shell almonds, AF production was relatively low (< 50 μ/kg) at all experimental conditions, with the three highest values being 47.4 μg/kg (at 20 °C and 10 days), 47.3 μg/kg (at 35 °C and 20 days), and 40.6 μg/kg (at 20 °C and 20 days) at 0.95 $a_w$.

For shelled almonds, however, AF production reached high levels at 0.95 $a_w$. At 27 °C and 0.95 $a_w$, AF production on shelled almonds increased throughout the study period (192.4, 273.1 and 325.4 μg/kg at day 10, 20 and 30, respectively). In contrast, at 20 °C and 0.95 $a_w$, AF production decreased on shelled almonds over the study period (324.3, 100.3 and 75.4 μg/kg at day 10, 20 and 30, respectively). There was also considerable AF production on shelled almonds at 35 °C and 0.95 $a_w$ (139.8 μg/kg at day 10 and 124.5 μg/kg at day 20). The increas-

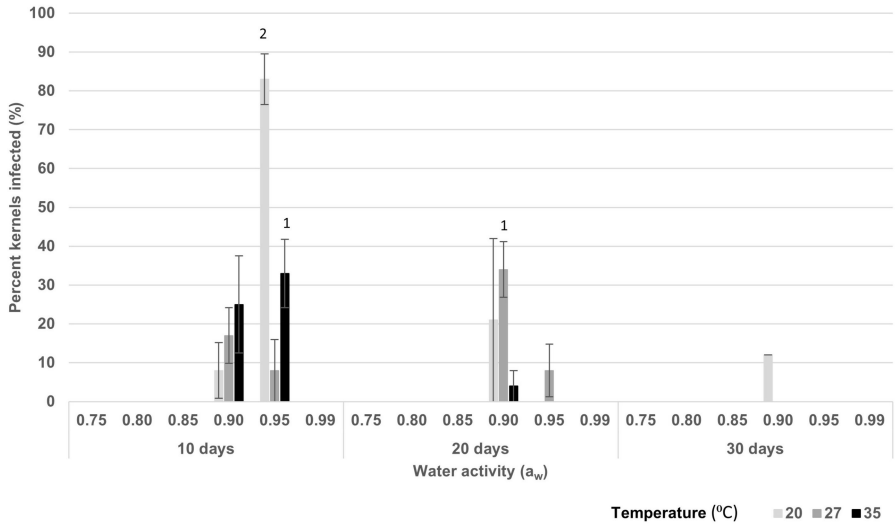


FIGURE 2 Growth of *Aspergillus parasiticus* on shelled almond kernels at various combinations of water activity and temperature at 10, 20 and 30 days of incubation. Error bars represent standard deviation. Numbers denote significantly different values within the dataset based on Tukey's multiple comparisons test.

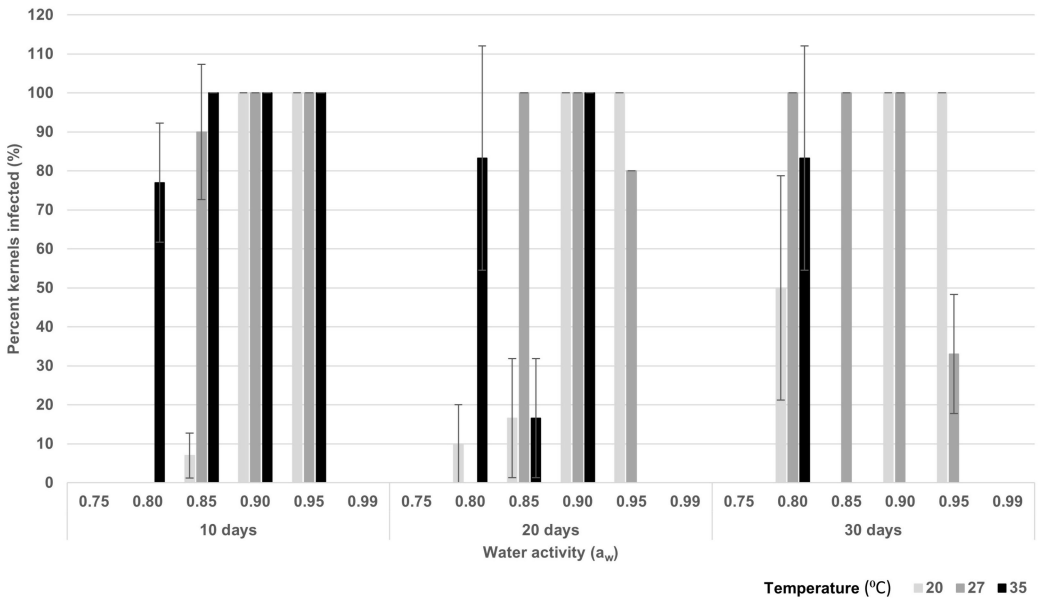


FIGURE 3 Growth of *Aspergillus parasiticus* on split almond kernels at various combinations of water activity and temperature at 10, 20 and 30 days of incubation. Error bars represent standard deviation.

ing trend of AF production over time at 27 °C but not at 20 °C and 35 °C may be because fungal growth persisted longer at 27 °C (there was still some growth at 20 days), so AF accumulation could continue at 27 °C for a longer time as compared to that at 20 or 35 °C. There was no AF production on shelled almonds at any of the other studied water activities.

### *Fungal growth and total aflatoxin production on split kernels*

In contrast to the in-shell and shelled almonds, *A. parasiticus* grew rapidly and well on split kernels under a wide range of conditions (Figure 3).

At day 10, there was 100% growth at 0.90 and 0.95 $a_w$ at all three temperatures. There was also substantial growth at 0.85 $a_w$ at 27 and 35 °C. The fungus was also able to grow at as low as 0.80 $a_w$ and 35 °C by day 10. At day 20, growth remained 100% on the split kernels at 0.90 $a_w$ at all three temperatures. Up to 30 days, growth remained substantial at 0.80 $a_w$ and 35 °C. Initially, the

TABLE 1 Analysis of variance (ANOVA) for the growth of *Aspergillus parasiticus* on split almond kernels

| Factor | F statistic | *P*- value | R-squared |
|---|---|---|---|
| Day 10 | | | 0.94 |
| Temperature (T) | 0.32 | 0.728 | |
| Water activity ($a_w$) | 88.15 | <0.001 | |
| T*$a_w$ | 11.5 | <0.001 | |
| Day 20 | | | 0.96 |
| Temperature (T) | 7.47 | 0.002 | |
| Water activity ($a_w$) | 118.19 | <0.001 | |
| T*$a_w$ | 37.31 | <0.001 | |
| Day 30 | | | 0.94 |
| Temperature (T) | 40.89 | <0.001 | |
| Water activity ($a_w$) | 48.61 | <0.001 | |
| T*$a_w$ | 21.82 | <0.001 | |

fungus did not grow at 0.80 $a_w$ and lower temperatures (20 and 27 °C), but by day 30, there was considerable growth at 0.80 $a_w$ at all three temperatures. However, the initial strong growth at 0.95 $a_w$ declined over time at higher temperatures (27 and 35 °C). Longer incubation time was needed for the growth of *A. parasiticus* at low water activity (0.80 $a_w$) and lower temperatures (20 and 27 °C). However, growth declined over time at the combination of high water activity (0.95 $a_w$) and higher temperatures (27 and 35 °C). There was no fungal growth on the split kernels at 0.75 $a_w$ at any of the study temperatures during the 30-day observation period. Also, there was no fungal growth on the three types of kernels at 0.65 $a_w$ over a six-month incubation period.

Analysis of variance revealed that water activity and the interaction of temperature and water activity (T*$a_w$) were the significant predictors of growth on split kernels ($P < 0.05$) from day 10 through day 30 (Table 1). The single factor temperature was not a significant predictor at day 10, however it became significant at days 20 and 30. The R-square values for all 3 models were >0.90, which means that the models explained at least 90% of the variation in the data (good model fit).

The fungus produced high levels of total AF in a wide range of water activities and temperatures on the split kernels (Table 2). At 0.80 $a_w$, AF production was initially very low at 20 and 27 °C, but it reached moderate levels (183.8 μg/kg) at 35 °C by day 10. After day 10, AF production at 0.80 $a_w$ steadily increased and by day 30, AF levels were >300 μg/kg at all three temperatures. Thus, incubation time had a critical role in AF production on split kernels at conditions of lower water activity. At 0.85 $a_w$, AF production was consistently high at 27 °C (> 300 μg/kg) and low to moderate at 35 °C. At 0.90 and 0.95 $a_w$, AF production was consistently high at 20 and 27 °C, however it was consistently low (<100 μg/kg) at 35 °C. At 0.99 $a_w$, AF production was very low for all temperatures and study periods. Thus, the combination of high water activity (> 0.90 $a_w$) and high temperature (35 °C) did not favour AF production on split almond kernels. In Figure 4, to show aflatoxin production at the same temperature and water activity conditions, data were averaged for days 10, 20 and 30 and statistically compared. For example, the data shows that at 27 °C, 0.85 $a_w$ and 0.90 $a_w$, aflatoxin production was statistically different from production at 35 °C and 0.85 $a_w$.

TABLE 2 Total Aflatoxin production (μg/kg) by *Aspergillus parasiticus* on split almond kernels at different combinations of water activity, temperature, and days of incubation

| Temperature (°C) | | 20 | 27 | 35 |
|---|---|---|---|---|
| Water activity ($a_w$) | Days | | | |
| 0.75 | 10 | ND[1] | ND | ND |
| | 20 | ND | ND | ND |
| | 30 | ND | ND | ND |
| 0.80 | 10 | ND | ND | 183.8 |
| | 20 | 189.2 | 16.6 | 299.5 |
| | 30 | 394.9 | 391.1 | 323.7 |
| 0.85 | 10 | 72.4 | 328 | 38.6 |
| | 20 | 318.2 | 331.3 | 124.1 |
| | 30 | 223.9 | 395.5 | 35.3 |
| 0.90 | 10 | 384.8 | 345.2 | 7.3 |
| | 20 | 335.3 | 319.1 | 7.0 |
| | 30 | 144.8 | 382.7 | 68.7 |
| 0.95 | 10 | 330.7 | 342.6 | 8.2 |
| | 20 | 352.6 | 366.4 | ND |
| | 30 | 360.2 | 318.2 | 15.4 |
| 0.99 | 10 | ND | ND | ND |
| | 20 | ND | 4.1 | 14.7 |
| | 30 | ND | 15.9 | ND |

[1] ND = not detected.

The contour plots reveal that the optimum conditions for AF production on split kernels were in the range of 0.90-0.95 $a_w$ and 20-27 °C throughout the study period (Figure 5). This is the range where consistently high AF production was observed. At 27 °C, there was a wide range of water activities (0.85 to 0.95 $a_w$) that resulted in consistently high AF production throughout the study period. At 35 °C only low water activity (0.80 $a_w$) resulted in high AF production.

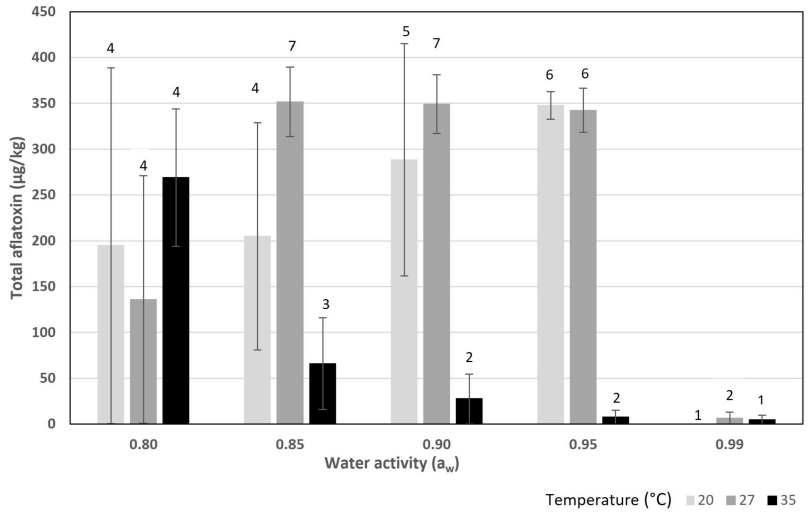


FIGURE 4 Total aflatoxin production on split almond kernels under different combinations of water activity and temperature (averaged over 10, 20 and 30 days). The error bars show standard deviations. The numbers above the columns indicate groups based on Tukey's multiple comparison test statistics, where 1 = A, 2 = AB, 3 = ABD, 4 = ABCD, 5 = BCD, 6 = CD, 7 = C. Groups that include the same letters are not statistically significant.

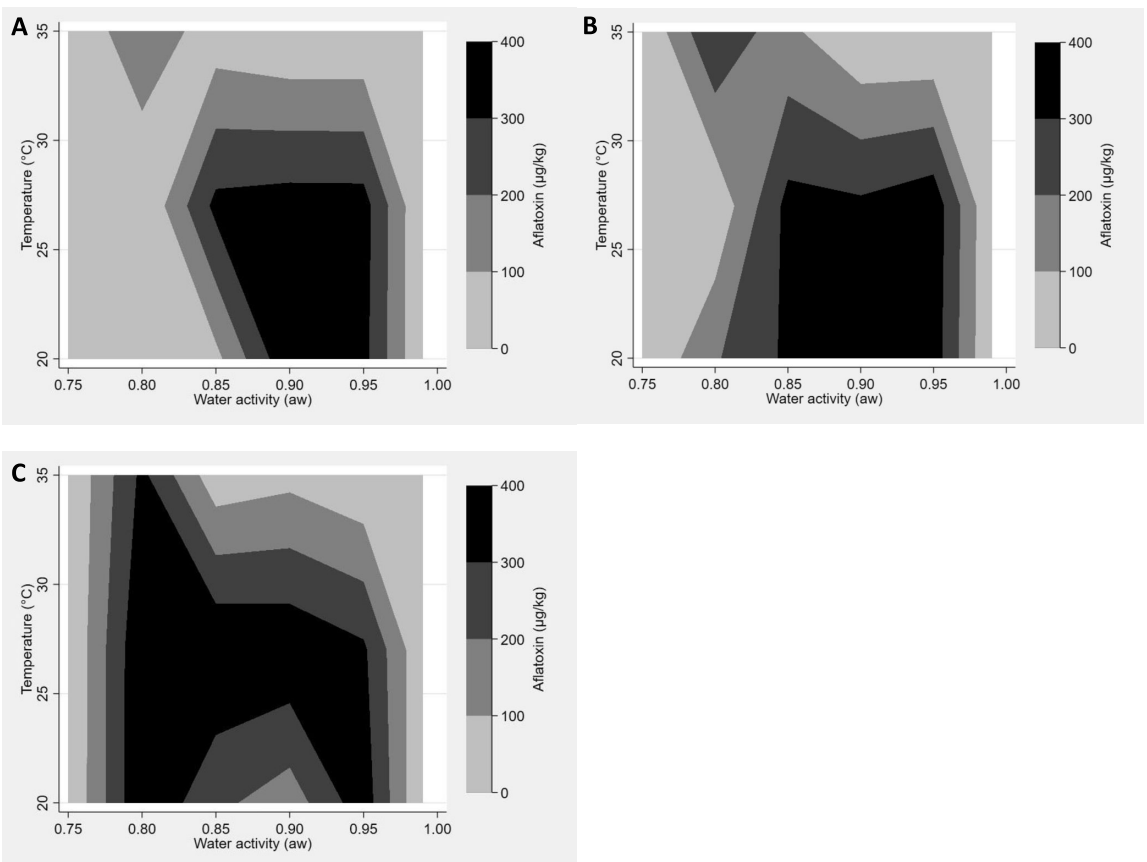


FIGURE 5 Contour plot of total aflatoxin production (µg/kg) by *Aspergillus parasiticus* on split almond kernels at 10 (A), 20 (B) and 30 (C) days of incubation.

## 4 Discussion

This study characterised the growth and total AF production of *A. parasiticus* on three different types of almond kernels under various incubation conditions. In addition to the effects of water activity and temperature, major differences were also noted in the ability of *A. parasiticus* to grow and produce aflatoxins depending on the type of kernel used as substrate. Furthermore, the incubation period was also found to play an important and complex role in the way the fungus grew and produced AF on the different kernel types.

Among the three kernel types, the in-shell kernels supported the least amount of growth and AF production overall. On shelled kernels, growth was also limited; AF production, however, reached high levels at 0.95 $a_w$. In contrast, the fungus grew rapidly and produced high levels of aflatoxins at a wider range of conditions on the split kernels. These results clearly show that exposing the nutrient rich inner almond meat allows rapid fungal growth to occur, while the almond shells and skins provided protection from infestation and AF production by *A. parasiticus*. The hard almond shell is composed mainly of cellulose, hemicellulose and lignin, which do not provide adequate nutrients for *A. parasiticus* to thrive (Li *et al.*, 2018). In addition, the thinner skin covering (seed coat) of the kernel of the shelled almonds also appeared to provide some protection from fungal infection. The almond skin contains biologically active molecules, including phenolic compounds, which may reduce or inhibit the growth of moulds (Garrido *et al.*, 2008). Numerous studies in the literature reported that intact almond shells and seed coats provided effective barriers against fungal invasion and mycotoxin accumulation (Moral *et al.*, 2022; Picot *et al.*, 2017). For example, a recent study showed that the presence of intact shells on almonds reduced AF production by *Aspergillus* spp. 100-fold (Moral *et al.*, 2022). Contamination with aflatoxigenic fungi may occur on damaged kernels that resemble split kernels. Sorting the almonds and removing the damaged or split kernels could be an effective way to reduce infestation and AF production by *A. parasiticus*.

On both in-shell and shelled kernels, fungal growth declined over the study period. This can be explained by the unsuitability of the substrate to support continued fungal growth. Alternatively, possible competition from other species of moulds might have hindered the growth of *A. parasiticus*. In the present study, hyphae of other fungal species, presumptively identified as *Rhizopus* spp. and/or *Aspergillus niger*, became clearly visible on the almonds, which may have suppressed the growth of *A. parasiticus*. These other fungal species also grew on the un-inoculated samples that served as controls for the experiments. Both *Rhizopus stolonifer* and *A. niger* commonly infect almond kernels and cause hull rot, which is an important disease affecting the almond industry (Lightle *et al.*, 2019). Our study suggests that the PPO fumigation used for pasteurisation of almonds does not eliminate these two hull rot pathogens, but it might reduce infestation. In our study, we observed the growth of *R. stolonifer* at or above 0.80 $a_w$ at all three study temperatures on in-shell, shelled and split almonds. Further studies are needed to determine the interaction of aflatoxigenic species (*A. parasiticus* and *A. flavus*) with *R. stolonifer* on almond kernels and its effect on aflatoxin production.

Despite the limited growth of *A. parasiticus* on shelled almonds, AF production reached high levels at 0.95 $a_w$ at the 3 temperatures in our study. Even on the in-shell almonds, AF production exceeded both US and EU regulatory limits at 0.95 $a_w$. Consequently, maintaining lower water activity (<0.90 $a_w$) for storage and transport of both in-shell and shelled almonds appear to be critical to avoid crop rejection due to AF accumulation by *A. parasiticus*.

*A. parasiticus* has been reported to grow in a variety of seeds and grains at a relatively wide range of water activities and temperatures. For example, Gizachew *et al.* (2019) found that *A. parasiticus* was able to grow on ground Nyjer seeds at 0.86-0.98 $a_w$ and at 20, 27 and 35 °C. The same study showed that on ground Nyjer seeds with low water activity (0.86 $a_w$), the fungus showed a lag phase with no visible growth for 5 to 10 days. This lag phase was also observed in our study on the split almond kernels, where the highest fungal growth was not observed until day 30 at relatively low water activity (0.80 $a_w$). In addition, *A. parasiticus* was also able to grow at a wide range of water activities (0.86-0.98 $a_w$) on ground flax seeds at 27 and 35 °C, as well as at 0.86-0.90 $a_w$ at 20 °C (Ting *et al.*, 2020). Mathematical modelling estimated that the minimum water activity for growth of *A. parasiticus* on black peppercorn was as low as 0.73-0.76 $a_w$ (Yogendrarajah *et al.*, 2016). On split almonds, *A. parasiticus* was not able to grow at 0.75 $a_w$, however it did grow and produce aflatoxins at 0.80 $a_w$.

Studies have also been conducted on the growth of *A. parasiticus* on artificial media. Peromingo *et al.* (2016) studied the growth of *A. parasiticus* (strains CECT2681 and CECT 2688) on meat-based and dry-cured ham-based agar and reported that the ideal condition for growth was at 0.95 $a_w$ and 25 °C; however, the study did

not investigate the effect of temperature above 25 °C. Another study (Schmidt-Heydt *et al*., 2010) found that *A. parasiticus* BFE96p grew most rapidly on yeast extract sucrose (YES) agar at 0.99 $a_w$ and 35 °C. In contrast, growth results on almonds indicated that *A. parasiticus* did not grow well at very high water activity (0.99 $a_w$).

Gallo *et al*. (2016) conducted studies on almond-based media using *A. flavus* ITEM 7828 and found that the fungus did not grow at 0.90-0.93 $a_w$ at 20 °C. In contrast, *A. parasiticus* was able to grow on inshell, shelled and split kernels under these conditions in the present study. Additionally, Gallo *et al*. (2016) reported that maximum growth and aflatoxin production occurred at 0.96 $a_w$ and 28 °C on the almond-based medium, while the optimum conditions for growth and AF production on split kernels were at 0.90-0.95 $a_w$ and 20-27 °C by *A. parasticus*.

The finding of ANOVA on split almond kernels showed that water activity was the main determining environmental factor in the growth of *A. parasiticus* during the first 10 days. With time, the role of temperature also became significant along with water activity in creating favourable conditions for *A. parasiticus* to grow. Therefore, controlling water activity and keeping it at or below 0.75 $a_w$ appeared to be the critical factor in risk mitigation for split almonds, especially in short term storage/transport.

Several transit studies using data loggers placed inside boxes of almonds indicated that the relative humidity levels inside the boxes remained in the range of 44.5 to 61.9%, and the temperature ranged from 12.3 to 30.7 °C, during shipping over the ocean (unpublished data). These relative humidity levels during ocean transit ensure a low water activity (<0.65 $a_w$) for the almonds. Our study showed that *A. parasiticus* was not able to grow and produce aflatoxins at 0.65 $a_w$ on almonds for a six-month observation period at any of the study temperatures. Consequently, environmental conditions during ocean transit are not favourable for the growth and AF production of *A. parasiticus* on almonds.

Previous studies indicated that *A. parasiticus* was also able to produce aflatoxins under a wide range of water activities (0.86 to 0.98 $a_w$) and temperatures (20, 27 and 35 °C) on ground Nyjer seeds (Gizachew *et al*., 2019) and on ground flax seeds (Ting *et al*., 2020). Furthermore, it was reported that *A. parasiticus* could produce aflatoxin in the range of 20-40 °C with $a_w$ > 0.90 on YES agar (Schmidt-Heydt *et al*., 2010). In our study, *A. parasiticus* could produce aflatoxins under a wide range of conditions on split almond kernels, but only at 0.95 $a_w$ on in-shell and shelled almonds. These findings indicate that whole kernel or in-shell almonds are not conducive to the growth and aflatoxin production of *A. parasiticus* up to 0.85 $a_w$ water activity.

In the present study, AF was detected in a similar range of temperatures and water activities that also supported fungal growth. In other words, there was a high positive correlation between the growth of *A. parasiticus* and AF production. The ideal conditions for both growth and AF production were in the range of 0.90-0.95 $a_w$ and 20-27 °C on split almonds. Also, the combination of high water activity (> 0.90 $a_w$) and high temperature (35 °C) resulted in a decline in both the growth and AF production on split almond kernels over the course of 30 days. The pooled data of the aflatoxin production for days 10, 20 and 30 showed that a higher temperature (35 °C) with water activity above 0.80 $a_w$ was not favourable for aflatoxin production compared to 20 and 27 ºC. At the same time, both fungal growth and AF production at 0.80 $a_w$ increased over the study period for the split kernels. Therefore, the effect of incubation time depended on the environmental factors.

In conclusion, the results of this study show the kernel types and conditions (0.90-0.95 $a_w$ and 20-27 °C) where the threat of spoilage due to *A. parasiticus* on almonds is high. The risk of infestation by *A. parasiticus* during storage and transportation can be reduced by sorting damaged kernels and maintaining low water activity (<0.75 $a_w$).

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**Authors' contribution**

BS and DG performed experiments, analysed data, wrote the manuscript; GH and TB supplied transit loggers' data, reviewed and edited manuscript. All authors have read and agreed to the published version of the manuscript.

**Conflict of interest**

GH is Associate Director of Food and Research Technology at ABC. TB is Director of Quality Assurance and

Industry Services at ABC. The article reflects the views of the authors and not necessarily those of the funder.

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