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Securing Rice Safety Through Innovative Radiative Heat Treatment and Proper Storage

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Food Science

by

Faith Achieng Ouma
Chuka University
Bachelor of Science in Biochemistry, 2020

August 2023
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

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ABSTRACT

Securing the microbial safety of rice is a rising priority within the food industry, especially when used as an ingredient to manufacture ready-to-eat, minimally-processed products. Mold contamination is typically the most problematic for rice because certain species of *Aspergillus* produce toxic secondary metabolites known as aflatoxins, rendering the grain unfit for human and animal consumption. The objectives of this study are to: (1) investigate the effectiveness of using high-power (915 MHz frequency), short-duration microwaves (MWs) to inactivate microbes on rough rice; (2) use a three-level screening design to determine which storage factors, such as temperature, relative humidity (RH), storage duration, rice moisture content (MC), and initial *A. flavus* spore concentration, significantly influence the growth kinetics of *Aspergillus flavus*, ergosterol, and aflatoxin B1 (AFBI) production in rice. A follow-up experiment was then conducted to assess the impact of significant factors on AFB1 and ergosterol production at extreme levels. Freshly harvested long-grain rough rice (RT 7321) with a MC wet basis (w.b.) of 21% and a bed thickness of 20 mm was exposed to MW powers of 16, 18, and 20 kW for varying durations (1, 2, and 3 minutes). The microbial load on treated and untreated samples was then determined using standard procedures. Rough, brown, and milled rice samples with different MCs (12%, 15%, and 18% w.b.) were used for the second objective. The rice samples, both autoclaved and non-autoclaved, were inoculated with different *A. flavus* spore concentrations (1×10^4 and 1×10^6 spores/mL) and incubated at different temperatures (20°C, 25°C, and 30°C) and RHs (75%, 85%, and 95%). After incubation period of 3, 9, and 15 days, *A. flavus* plate count, ergosterol and AFB1 production were measured. Throughout all storage durations, *A. flavus* growth was optimal at 30°C and 95% RH. AFB1 production was dependent on rice fraction and storage conditions, with brown rice having the highest concentration (9.198 μg/g) after 15 days of incubation at 30°C and 75%

RH. However, during the latter part of the study, it was observed that AFB1 concentrations in brown rice inoculated with 1×10^6 spores/mL of *A. flavus* and stored at 30°C and 95% RH initially increased after 20 days and then decreased towards the end of the storage period (60 days). The results are expected to help understand the application of MW technology to mitigate toxicity-related problems associated with the presence of microbes in rice and identify proper storage conditions that can minimize the risk of aflatoxin contamination, thereby improving rice safety.

ACKNOWLEDGMENTS

I would like to express my gratitude to the divine Almighty God for bestowing his grace upon me throughout this entire journey. I extend heartfelt thanks to my advisor, Dr. Griffiths Atungulu, for being an exceptional mentor and providing me with the opportunity to unleash my potential in all capacities.

I also wish to convey my sincere appreciation to my committee members, Dr. Burt Bluhm and Dr. Philip Crandall, for their invaluable feedback, which greatly contributed to the success of this work. Special mention goes to Dr. Andy Mauromoustakos for his assistance in analyzing the data for this project.

To all the members of my laboratory, I am deeply grateful for the pivotal roles each of you played in the completion of my project. I would like to specifically thank Dr. Kaushik Luthra for your enthusiastic guidance and encouragement to keep pushing, as well as Abass Oduola for your selfless assistance throughout the project.

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TABLE OF CONTENTS

INTRODUCTION

With increased demand for diversity in the food market driven by consumers, thriving for a healthy lifestyle has impelled the development of new rice products. Formulations designed using rice flour combined with other ingredients show potential benefits for individuals on gluten-restricted diets (Wesley et al., 2021). Typically, rice is predominantly consumed in white rice form, particularly in Asian countries, where it serves as a primary source of starch (Lai et al., 2015). Even though rice is regarded as a low-moisture grain, as per Rifna et al. (2019), low-moisture foods are presumed to be safe from microbial invasion owing to the fact that they have a low water activity of less than 0.7, but they are still prone to contamination, which results in their spoilage (Blessington et al., 2013). Yeast, molds, and bacteria are among the microorganisms known to live naturally on freshly harvested rice (Atungulu et al., 2014). The presence of these natural contaminants may render the grains unsafe for consumption because they can produce a range of potentially toxic substances, such as aflatoxins, which are of most concern to health. Mold contamination, specifically by toxigenic fungus *Aspergillus flavus*, is a major concern for rice producers, processors, and consumers, having been identified as the most commonly observed microbe colonizing the surface of rice grain (Mohamadi et al., 2017).

The contamination of rice is largely due to improper drying after harvest, although there are some species that target the crop while it is still growing in the field. The main abiotic factors found to deteriorate rice quality during storage are temperature and moisture (Atungulu et al., 2016). While drought and high temperatures are the most significant influences on preharvest fungal contaminants (Asghar et al., 2014). When rice is harvested at initial moisture contents (MC) of 16 -24%, it is immediately dried to about 13–14% MC (w.b.); this facilitates maintenance of the optimal quality during long-term storage (Atungulu et al., 2016). Moreover, since the crop is

harvested seasonally, it is therefore imperative that the post-harvest processes such as drying, storage, and processing are critical to ensuring its safety and availability in the future (Coradi et al., 2022). In the United States, approximately 80% of harvested rice is dried using high-temperature and cross-flow drying systems, while the other 20% is dried on the farm using in-bin drying systems (Schluterman and Siebenmorgen, 2004). However, the drawback of in-bin drying includes implications caused by weather conditions, which may delay drying for weeks, especially on grains at the top layers. This longer time elapsing after harvesting due to prolonged drying creates a conducive environment for the proliferation of the microbes. Consequently, bacterial and fungal contaminants can lead to organoleptic changes in the rice, such as discoloration, mustiness, and dry matter losses due to excessive respiration.

Therefore, this study first specifically seeks to explore microwave technology in rice drying to determine the effect of high-power microwaves (915 MHz) with short-time heating on the inactivation of aerobic bacteria and fungi. For the second part, the study endeavored to understand which storage factors (storage temperature, relative humidity (RH), storage duration, rice moisture content (MC), and initial *A. flavus* spore concentration) significantly influenced the growth of *Aspergillus flavus* in different rice fractions (rough, brown, and milled rice).

EFFECT OF HIGH-POWER SHORT-DURATION MICROWAVE HEATING ON INACTIVATION OF MICROBES ON ROUGH RICE

ABSTRACT

As rice consumption increases, ensuring its safety has become a priority for the food industry. To address this, the industry is exploring the use of a single-pass microbial inactivation treatment at the rough rice stage. This study aimed to evaluate the efficacy of a high-power, short-duration microwave treatment (at 915 MHz frequency) for inactivating microbes on rough rice while maintaining its quality. Freshly harvested rough rice with high moisture content (21.2% wet basis) and a 20 mm bed thickness was exposed to microwaves at 16, 18, and 20 kW for durations of 1, 2, and 3 minutes. Treated and untreated samples were analyzed for microbial load using standard procedures, including total aerobic count (TAC) and total fungal count (TFC) enumeration. The results indicated that the highest microwave power (20 kW) and longest exposure duration (3 minutes) produced the greatest reduction in TAC and TFC, up to 1.21 CFU/g and 5.01 CFU/g log reductions, respectively. A single pass treatment with 20 kW microwave power for 3 minutes was effective in reducing fungal growth on a thin layer of rough rice, while higher bacterial reduction may require longer duration or higher power. These findings suggest that high microwave powers with an exposure duration of about 3 mins can be a viable option for significant inactivation of microorganisms on rough rice.

Keywords. Rice consumption, safety, single-pass microbial inactivation, microwave treatment

INTRODUCTION

Rice (*Oryza sativa*) accounts for more than 20% of the calories consumed by over 3.5 billion people worldwide. According to the most recent Organization for Economic Co-operation and Development-Food and Agriculture Organization of the United Nations (OECD-FAO) Agricultural projections, global rice production is expected to increase by 11.4% by 2030, reaching 567 million tonnes (OECD-FAO, 2021), required to feed the growing global population (Bin et al., 2022) . Furthermore, due to its nutritional properties, the development of new rice products, such as gluten-free rice flour, has sparked interest in rice utilization in the food industry (Luthra and Sadaka, 2020; Wesley et al., 2021).

Post-harvest processes such as drying and storage are critical to ensuring the safety of rice (Müller et al., 2022). Rice is harvested with an initial moisture content (MC) of 16-24% wet basis (w.b.) that needs to be immediately dried to 13-14% MC (w.b.). This helps maintain the optimal quality during long-term storage because the high moisture content creates an ideal environment for microbial proliferation (Atungulu et al., 2016; Luthra and Sadaka, 2021). *Aspergillus flavus* has been identified as the most common microbe found on the surface of rice grains (Mohamadi et al., 2017). These natural contaminants may render the grains unfit for human consumption because they can produce a variety of potentially toxic substances, including aflatoxins of particular health concern.

Both chemical and thermal technologies are effective in inactivating microbes in food products. However, the use of chemical treatments that require fumigation, such as ozone, chlorine, and ethylene oxide, are prohibited in the European Union and the United States due to environmental and health concerns (Shirkole et al., 2021). Compared to non-thermal technologies such as pulsed light, high-pressure processing, and cold plasma, thermal technologies require high treatment intensities to reduce microbial load (Deng et al., 2019). On the other hand, conventional

thermal treatments use high heat to denature microbial DNA but at the expense of affecting nutritional and physicochemical properties of products, such as the loss of desirable bioactive and antioxidant compounds (Rifna et al., 2019). In cases where low temperatures are used for conventional heat treatments, often to maximize rice milling quality, it is impossible to achieve the accepted 5 log reduction standard by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF; Smith and Atungulu, 2017, 2018). For this reason, the search for alternative technologies that could decontaminate and preserve nutritional and sensory properties led to the introduction of new technologies such as microwave heating, infrared heating, ionizing radiation, and ultraviolet rays.

Microwaves are a type of electromagnetic radiation with wavelengths ranging from 1 mm to 1000 mm with a frequency that falls between 300 GHz and 300 MHz, respectively. Microwave heating as a drying technique of rough rice gained popularity (Le et al., 2014), particularly after Atungulu et al. (2016) reported that the technology could achieve one-pass drying at specific energies of around 600 kJ/kg followed by tempering, resulting in a significant reduction of MC to a final MC of 14% to 16% (w.b.). Typically, a MC of about 12.5% (w.b.) is considered safe for long-term storage, acting as a critical control limit that helps eliminate potential hazards brought on by microbes in rice. Microwave technology has been used successfully for microbial decontamination in food products (Eliasson et al., 2015; Jeevitha et al., 2016). The mechanism of action, which can be traced back to the heating impact of radiation, causes damage to the microbial cell wall, genomic DNA damage, and cytoplasmic protein agglomeration, which leads to the microorganism's gradual death (Skowron et al., 2022). The energy fluxes associated with convectively heated air have proven insufficient (Wilson et al., 2016). From an industrial standpoint, incorporating high MW power with short heating durations increases energy fluxes.

Besides decontamination, it also targets to reduce drying costs through proper energy management (Chojnacka et al., 2021). Furthermore, a MW drying approach for rough rice combined with a tempering step can help increase head rice yield to greater than 65% and overall quality, compared to the current average head rice yield of 58% from convective drying based on laboratory drying simulations (Atungulu & Smith, 2020). Considering the current market price of milled rice, a 7% increase in head rice yield translates to an estimated economic benefit of approximately $70.56 per acre.

Previous studies that dealt with the use of microwave as a drying and decontamination method employed a frequency of 2450 MHz (Kaasova et al., 2002; Liu et al., 2016; Vadivambal et al., 2007) and reported that there are limitations to the frequency to penetrate thick grain beds that exceeding 15 cm (Kumar, 2015). Conversely, a microwave set to operate at 915 MHz has a higher large-scale decontamination potential due to its penetration ability, which is three times greater penetration than 2450 MHz (Smith and Atungulu, 2018). Thus, the specific objective designed for this study is to investigate the effect of increasing MW heat intensities with heating durations on the inactivation of aerobic bacteria and fungi in rough rice.

MATERIALS AND METHODS

RICE SAMPLES

Freshly harvested long grain rice samples, variety RT 7321, were used in this study. The initial MC of the rough rice was 21.2% (w.b.). The obtained samples were cleaned using dockage equipment (MCI kicker dockage tester, mid-continent industries inc, Newton, KS). The equipment separated weed, chaff, grain, and any other foreign material other than rice grain using appropriate sieves. The cleaned rice was placed in tubs and stored in a laboratory cold room set at 4°C for

about 48 hrs. Before the beginning of the experiments, all the samples were obtained from the cold room and allowed to equilibrate at room conditions of 25°C until the period of use. The MC of the samples was determined using an AM 5200 grain moisture tester (PERTEN instruments, Hägersten, Sweden).

MICROWAVE HEATING

An industrial MW system (Applied microwaves technology inc, Cedar Rapids, IA) was used in this study. The system consisted of a transmitter, a waveguide, and a MW heating zone (oven), which was set to operate at a frequency of 915 MHz. The MW generator employed a high-powered vacuum tube and functioned as a self-excited MW oscillator, converting high-voltage electric energy into MW radiation. The waveguide in the system was designed as a rectangular pipe through which the electromagnetic field propagated along its length. This pipe served to convey the generated MW from the magnetron into the heating cavity, where the rough rice samples were positioned for thermal treatments. The MW system details can be found in Olatunde et al. (2017).

The implications of MW power levels (16, 18, and 20 kW) and heating durations (1, 2, and 3 min) on the microbial load reduction for rice bed thickness at 20 mm were studied. Twenty-seven total experiments were conducted as described in Table 1. A sample of 3 kg of rough rice was weighed and placed into the hopper to be fed on the conveyor belt and treated at various power levels that were heating from top to bottom with airflow from bottom to top for the specified durations. The conveyor belt's speed was 48 cm/minute, 24 inches/minute, and 16 inches/minute for 1, 2, and 3 minutes, respectively. Specific energy (energy supplied per unit mass of rice sample) used for various treatments ranged between 320 kJ/kg and 1200 kJ/kg. Following treatment, the rice samples were transferred to tubs, sealed airtight, and stored in a 4°C laboratory cold room.

MEASUREMENT OF RICE SAMPLE MOISTURE CONTENT

Before microbial analysis, the samples were allowed to equilibrate in a laboratory environment (25°C for 24 hours). After equilibrating, the MC was determined using the AM 5200 grain moisture tester and then 10 g of the treated sample was taken out for microbial analysis. Control samples were not treated with MW but were gently dried to a MC of 12.5% (w.b.) in an environmental-controlled chamber (air temperature of 25°C and relative humidity of 56%).

MICROBIAL ANALYSES

The microbial enumeration was carried out using standard procedures to determine the total microbial load. The standard procedures for fungal and bacterial identification, plating and counting were adapted from Ranalli et al. (2002) and Smith et al (2018) with some slight modification. In a 1 L volumetric flask, 34 g of KH_2PO_4 was dissolved in 500 mL of water to make phosphate-buffered dilution water. Using 1 M NaOH solution, the pH of the solution was adjusted to 7.2. Then, in the volumetric flask, distilled water was added to make a stock solution with a volume of 1 L. The stock solution was then autoclaved at 121°C for about 20 minutes to ensure sterility before use. A 10 g sample of the rough rice was weighed and placed into a sterile stomacher bag. Then, 90 mL of sterile phosphate-buffered dilution water was added to the stomacher bag and masticated. A lab masticator (Silver panoramic, iUL, SA, Barcelona, Spain) set at 240 s and 0.7 strokes/s was used to dislodge the microorganism by ensuring that the rough rice samples were pulverized into powder for microbial analysis when mixed with dilution water.

Serial dilutions were carried out by mixing 1 mL of the original mixture in the stomacher bag (first dilution 10^{-1}) with 9 mL of sterilized phosphate-buffered dilution water in a test tube (second dilution 10^{-2}) and so on until the sixth dilution (10^{-6}) was made.

MICROBIAL PLATE COUNT

Fungi and bacteria counts were counted using 3M Petrifilm fungal Count Plates and 3M Petrifilm Aerobic Count Plates (3M Microbiology products, Minneapolis, MN). The plates were placed in the biosafety cabinet. The top film of the plate was carefully lifted and a P1000 micropipette (Finnpippete F2, Thermo fisher scientific, inc, Vantaa, Finland), placed perpendicular to the plates, was used to transfer 1 mL of the sample solutions onto the center of the 3M Petrifilm plates. The top film was then gently lowered. The center of a plastic spreader was placed on the plates to align with the centers of the plates. Light manual pressure was then applied to the plastic spreader to ensure the even distribution of the inoculum on the Petrifilm plate. The gel was allowed to solidify for 1 min. The inoculated Petrifilm plates with clear sides up were stacked to a maximum of 20 units and were placed in an incubator (Thelco model 4, Precision scientific instrument, inc., Buffalo, NY) at 25°C for 120 hrs (fungi) and at 35°C for 48 hrs (bacteria), before counting. After the incubation periods, the colony forming units (CFU) on each plate were counted. Fungal colonies on the plates appeared blue, black, yellow, or green on the plates, whereas bacteria colonies appeared red with a regular shape.

The colony-forming unit per gram of rice (CFU/g) for each sample was obtained using equation 1.

$$T_{cfu} = \frac{P_{cfu}}{D_r}$$

where

T_{cfu} = total colony forming units per gram of rice (CFU/g),

P_{cfu} = colony forming units counted on the plate per gram of rough rice, and

D_r = dilution rate 10^{-1} to 10^{-6} durations.

STASTISTICAL ANALYSES

The data was statistically analyzed using the statistical software JMP Pro 16.0. (SAS institute, Cary, NC). Analysis of variance (ANOVA) was used to determine the interaction effects of MW powers and heating durations on the log reduction response of aerobic bacteria and fungi with post-hoc means separation tests, including Tukey's honest significant difference (HSD) test for comparing more than two means and the t-test for comparing just two means. All the tests were significant when $p < 0.05$.

Table 1. Experimental design

Factor	Levels	Number of experiments
Power level (kW)	16, 18 and 20	
Duration (min)	1, 2 and 3	3 × 3 × 3 = 27
Replication	1, 2 and 3	

RESULTS AND DISCUSSION

The initial population means of aerobic bacteria and fungi in control samples were 7.88 log CFU/g and 7.22 log CFU/g, with standard deviations of 0.3 and 0.2, respectively. Atungulu et al. (2016) investigated microbial growth kinetics on freshly harvested rough rice, different cultivars, from different locations in the United States, and found average levels of aerobic bacteria and fungi plate count to be 8.19 and 7.75 log CFU/g, respectively. The ANOVA analysis revealed that MW power level and heating duration had a significant effect ($p < 0.05$) on both bacterial and fungal load. However, the interaction between the two was only significant for fungal load (Table 2). This result indicated that the effect of the main factor MW on fungal reduction was dependent on the level of the other factor, heating duration.

Root means square error (RMSE) is used to validate the model by estimating the standard

deviation. In our statistical analysis, the RMSE for aerobic bacteria and fungi was 0.1886 and 0.1563, respectively. Furthermore, our model explained approximately 85% (R squared = 0.85) and 95% (R squared = 0.95) of the variation in aerobic bacteria and fungi responses, respectively. A higher R squared is preferable because it demonstrates how well the model fits the data and allows for meaningful conclusions about how changes in predictor variables affect the response variable. In this case, our predictor variables were MW powers and heating duration, while our response variables were aerobic and fungal log reduction count.

Table 2. Summary table showing effects of microwave power, heating duration, and their interaction on aerobic bacteria and fungal response (Log CFU/g).

Source	Aerobic bacteria	*Fungi*
	***p*-value**	***p-value***
Power (kW)	<0.0001*	<0.0001*
Heating duration (min)	<0.0001*	<0.0001*
Power (kW) * Heating duration (min)	0.0636	<0.0001*

*p < 0.001 indicates significant effect of the main factor and interaction on aerobic bacteria and fungal response.

EFFECT OF MICROWAVE POWER ON MICROBIAL LOAD

Increasing the microwave power resulted in a significant increase in the log reduction for both aerobic bacteria and fungi, as shown in Figures 1 and 2. Fungi achieved higher mean log reductions at 20 kW than aerobic bacteria, 3.5 log CFU/g and 1.03 log CFU/g, respectively. The low log reduction of aerobic bacteria compared to fungi, by contrast, could be attributed to the heat resistance of predominant bacteria and bacterial spores to degradation. Zhao et al. (2021) discovered that bacterial contaminants form biofilms around tofu coagulates, significantly increasing their heat tolerance to microwave treatment. Furthermore, higher heat fluxes are produced by the highest MW power (20 kW), as evidenced by its ability to significantly reduce the microbes. Besides that, there were no statistically significant differences in aerobic log reductions between MW power levels of 18 kW and 16 kW.

On the other hand, fungal log reduction responses to the three MW power levels (16 kW, 18 kW, and 20 kW) were all significantly different (Fig 2). It should also be noted that fungal responses to MW power levels of 18 kW and 16 kW differed significantly. This observation was in contrast to that for aerobic bacteria, which showed that on treatment with the same MW powers across all the heating durations, they were not significantly different (Table 2). Earlier researchers have shown that the efficacy of MW powers on the inactivation of microbes is dependent on various factors, including specific energy, rice bed thickness, and heating duration. Smith and Atungulu (2018) discovered that rice bed thickness was a crucial parameter in the response of aerobic bacteria while power was the most significant factor in the fungal response using a 915 MHz frequency at power levels of 5, 10, and 15 kW. In their study, different rice bed thicknesses (5, 10, and 15 cm) resulted in higher aerobic counts at the top layers than at the bottom layers. In this study, a bed thickness of 2 cm was chosen to maintain uniform decontamination at the bottom and top layers.

EFFECT OF HEATING DURATION ON MICROBIAL LOAD

The most general way of evaluating microbial inactivation is by determining the survival rate of microbes based on treatment time and intensity (Álvarez et al., 2003; Puértolas et al., 2009). However, when determining the efficacy of any sterilization technology, it is also necessary to quantify the amount of energy being transferred per unit mass of samples being treated (Rodriguez-Gonzalez et al., 2015). This addition provides insights to the magnitude of energy input required to inactivate micro-organisms. Overall, duration as a factor was statistically significant both for aerobic bacteria and fungal log reduction. More thermal energy was supplied to the rice as the duration increased, allowing the temperature to rise. As a result, more microbial organisms were rendered inactive. However, for aerobic bacteria at 16 kW and 18 kW, there was some thermal

resistance developed after 2 minutes of exposure (640 kJ/kg and 720 kJ/kg, respectively), which reduced the log reduction after 3 minutes (960 kJ/kg and 1080 kJ/kg, respectively) compared to that after 2 minutes (Fig 1). This observation resulted in a lower overall reduction of aerobic bacteria than fungi. The dose-response curve depicted in figure 4 better illustrates the disparity, demonstrating a higher level of survival among aerobic bacteria with respect to the specific energy applied. It is apparent that bacteria were more resistant to the treatments than fungi, making them more resilient to inactivation. According to Russell et al. (2003), bacteria have evolved several mechanisms to help them mitigate stress responses, including the production of heat-shock proteins to withstand high temperatures. Similarly, Mackey and Derric (1986) discovered that bacterial stress resistances response to heat resistance increased after short-term exposure to elevated temperatures.

The maximum and minimum reductions for aerobic bacteria were 1.22 and 0.21 log CFU/g at 18 kW (2 min) and 16 kW (1 min), respectively which corresponded to a specific energy of 720 kJ/kg and 320 kJ/kg). For fungi, the maximum and minimum reductions were 5.01 and 0.99 log CFU/g at 20 kW (3 min) and 20 kW (1 min), respectively which corresponded to a specific energy of 1200 kJ/kg and 400 kJ/kg). And since the fungal reduction achieved at 18 kW for 3 minutes (1080 kJ/kg) of exposure was statistically similar to that achieved at the maximum power level of 20 kW for 3 minutes (1200 kJ/kg) of heating (Fig 2), the energy used at 18 kW can be used to achieve approximately 5 log reductions. This reduction in fungal survival was observed as specific heat energy increased as shown in Fig 3. A previous study by Eliasson et al. (2015) evaluated bacterial load on paprika powder (150 g) after MW treatment at a power level of 650 W for 20 minutes and reported a 4.8 log reduction. The specific energy use was 5200 kJ/kg. Smith and Atungulu (2018) determined that the power of 12.26 kW and heating duration of 6.67 min were

preferred for optimum inactivation of aerobic bacteria for 7.14 kg of rice. The specific energy use was 687 kJ/kg. In this study, the specific energy used for treating rough rice at 20 kW for 3 min of heating duration was 1200 kJ/kg. This amount of energy led to approximately 5.01 log reductions for fungi. No study reported a significant amount of fungal or bacterial log reductions for rough rice at the heating duration of three or fewer minutes with the specific energy use of 1200 kJ/kg.

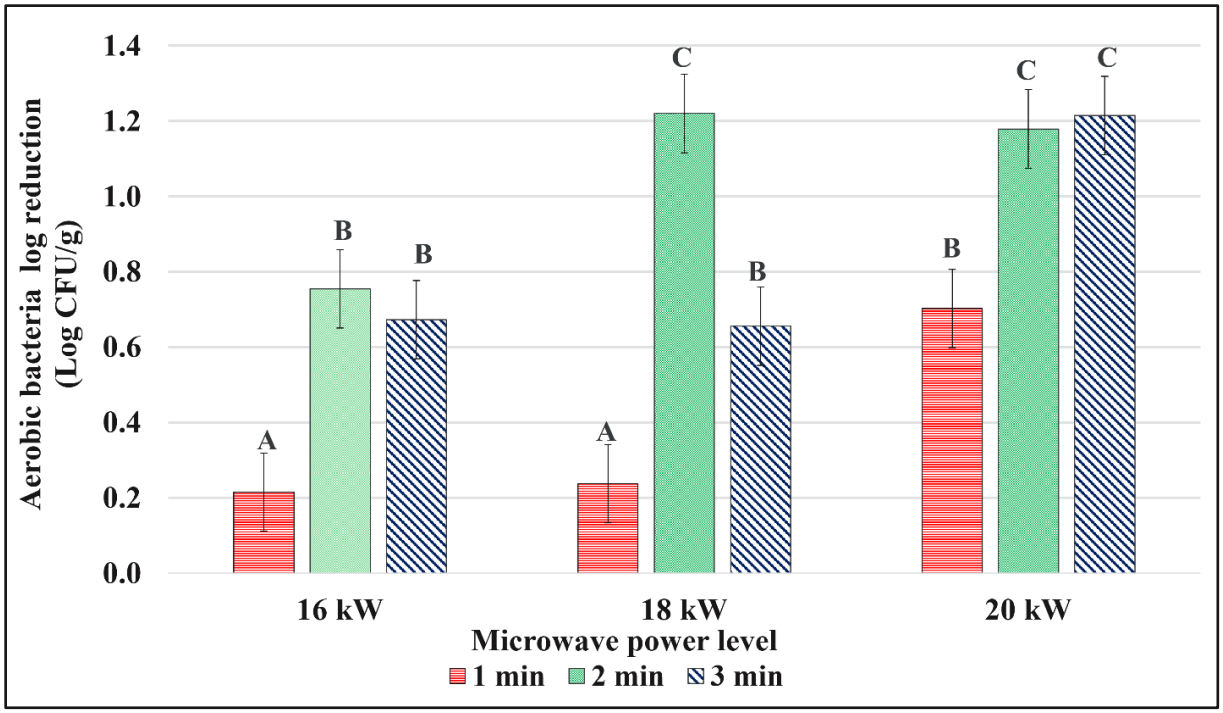

Figure 1. Effects of increasing microwave-power intensities on aerobic bacteria log reduction. (Bars not having the same letter signify a significant difference at a 95% level of confidence).

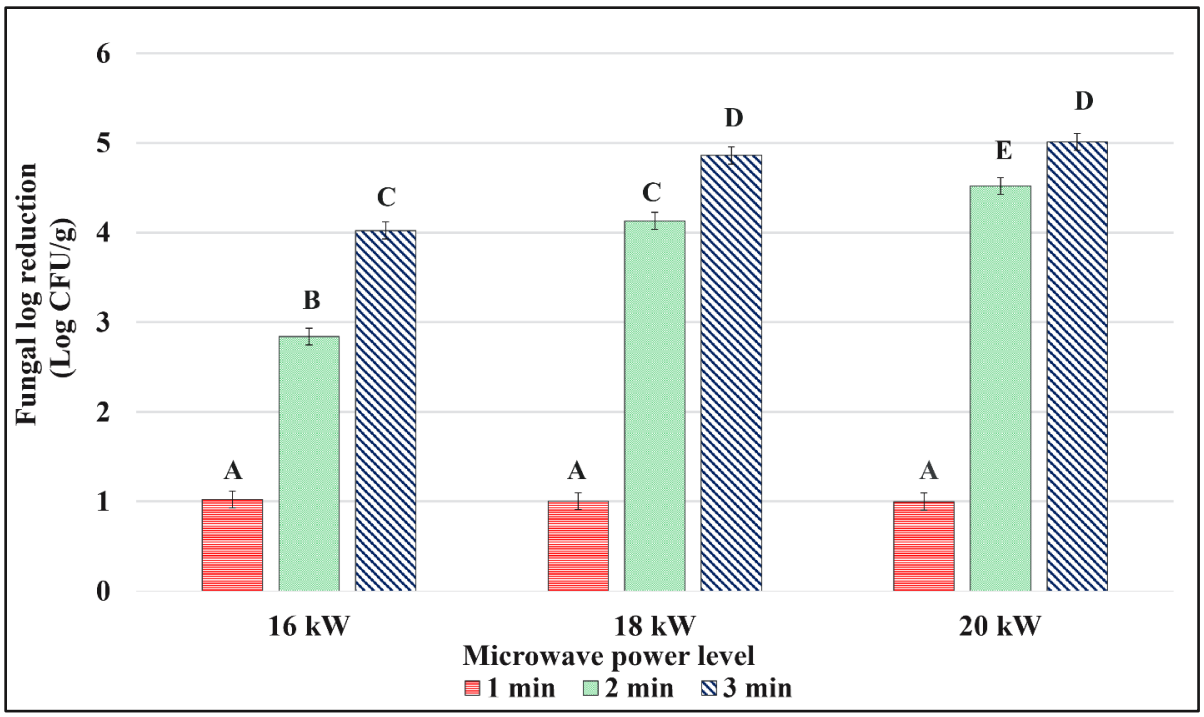

Figure 2. Effects of increasing microwave-power intensities on fungal log reduction. (Bars not having the same letter signify a significant difference at a 95% level of confidence).

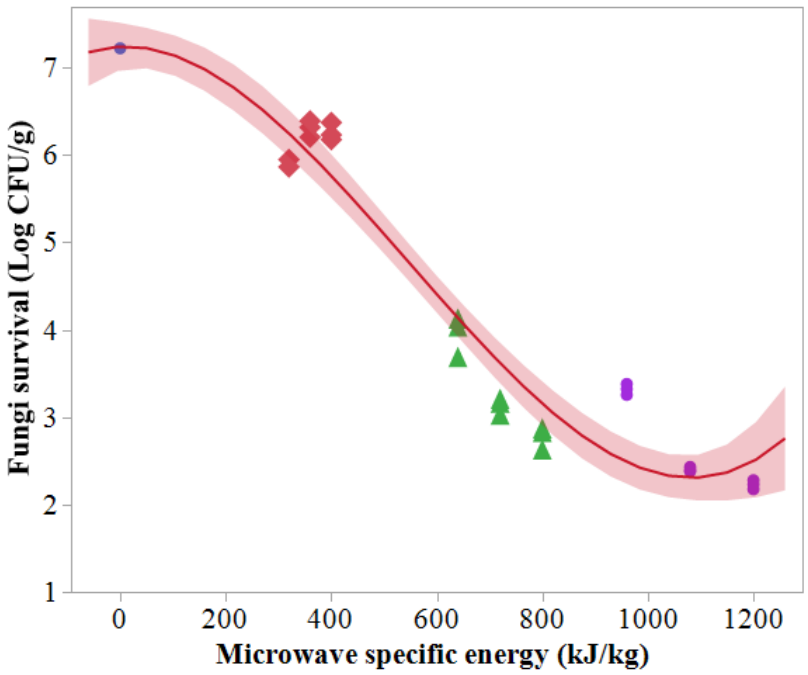

Figure 3. Dose response curve for fungi survival (CFU/g) as function of the applied MW specific energy (kJ/kg)

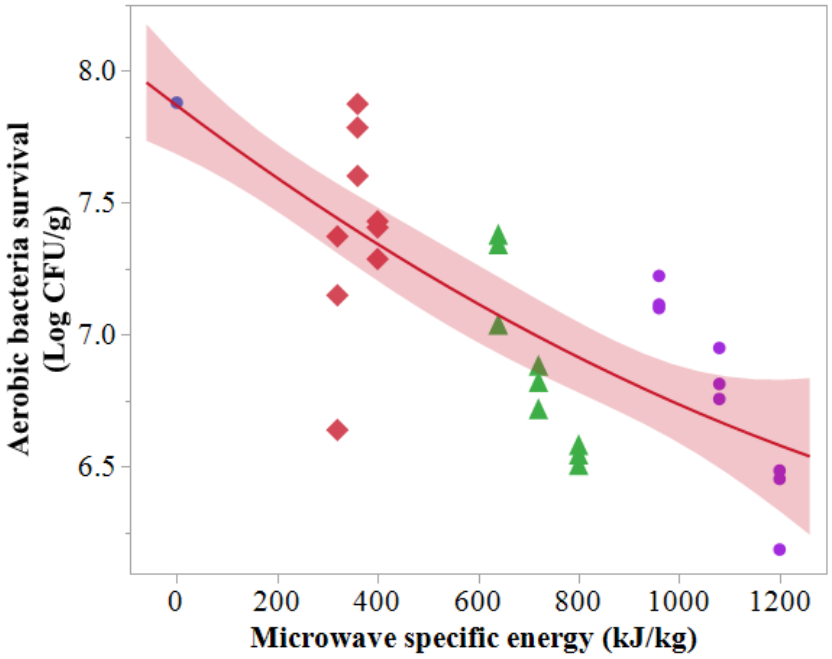

Figure 4. Dose response curve for aerobic bacteria survival (CFU/g) as function of the applied MW specific energy (kJ/kg)

OPTIMIZATION OF THE PARAMETERS

In an industrial setting, because multiple variables are being studied at the same time optimization is critical. For this reason, a decision on the best operating conditions to achieve the best overall performance would be considered. Using the red vertical lines shown in Figures 3 and 4, the prediction profiler option in JMP Pro 16.0 software was used to optimize MW power and heating duration factor settings to maximize the desired effects on aerobic bacteria and fungi responses. This tool makes optimization possible by allowing for simultaneous changes of the factors one at a time until the desired value is attained. The slopes of the lines for each predictor (MW and heating duration) reflect the model coefficients. The optimal setting was determined to be a MW power of 20 kW and a heating duration of 3 minutes, which would result in a log reduction of approximately 1.12 log CFU/g for aerobic bacteria and 4.98 log CFU/g for fungi, respectively (Figures 3). The geometric mean of desirability is represented on the vertical axis. For example, a desirability of 0.850833 indicates that approximately 85% of the aerobic bacteria and fungi log reduction optimization goals were met. The predicted values after parameter optimization and the data from our experiment did not differ significantly because they were within the confidence interval values shown in the brackets. Based on our data, as shown in Figures 1 and 2, after 3 minutes of heating with 20 kW, we achieved approximately 5.01 log CFU/g and 1.21 log CFU/g, respectively. Since the responses of different microbes differ depending on treatment parameters such as MW power, specific heat energies, and rice bed thickness, future research should focus on investigating the effect of these various parameters on specific microbes. Furthermore, Ueda and Kuwabara (1988) found that removing the hull and bran decreased the population of microbes in rice fractions. Investigating the effects of MW power levels, heating

duration, specific heat energies, and rice bed thickness on microbial responses in different rice fractions would thus be beneficial in maximizing microbial inactivation.

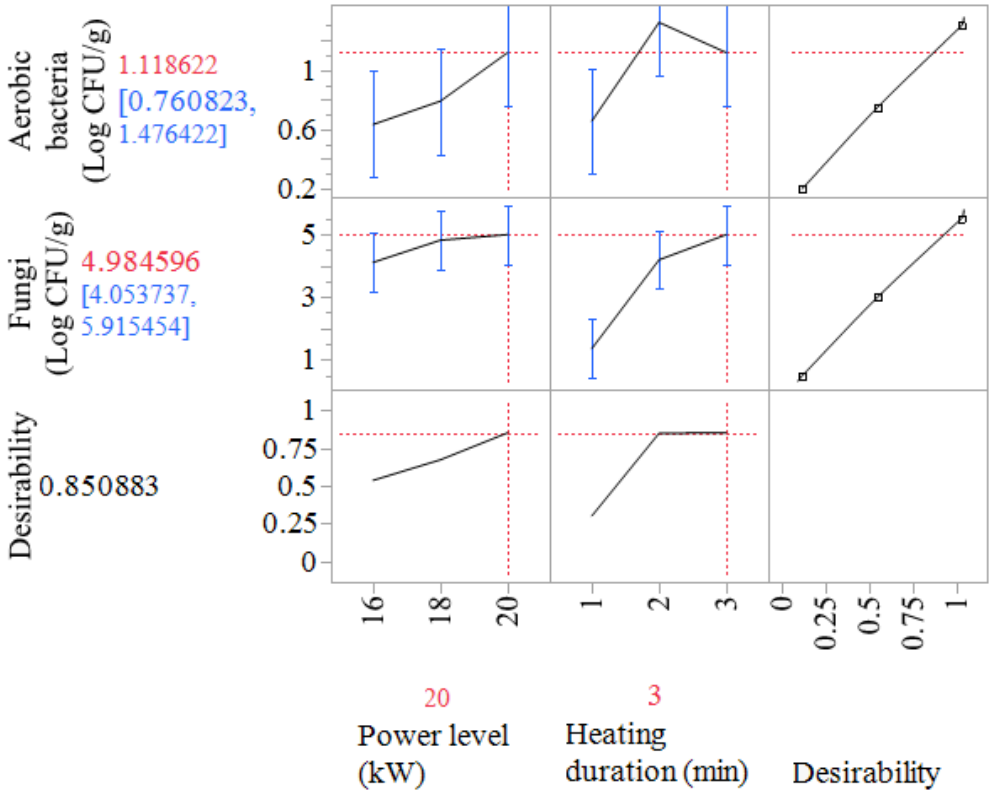

Figure 3. Prediction profiler showing optimum microwave (MW) power and heating duration level settings and corresponding aerobic bacteria and fungi log counts. In brackets represents 95% confidence interval for the average aerobic bacteria and fungi log reduction.

CONCLUSIONS

The results demonstrate that increasing microwave power and heating duration resulted in a significant increase in microbial load reduction in rough rice samples. In general, the selected 915 MHz MW had an impact on both fungal and aerobic bacterial counts. However, a power of 20 kW and a heating duration of 3 minutes, corresponding to a specific energy of 1200 kJ/kg, resulted in a greater log reduction in fungi (log 5.01 CFU/g) than aerobic bacteria (log 1.2 CFU/g). A longer heating duration or higher power may be required to achieve a substantial aerobic bacterial

log reduction. The use of single-pass continuous microwave treatment for a short duration could be a promising technology for microbial decontamination of rough rice. Nevertheless, more research needs to be done on optimizing bacterial reduction in a short period of time. This will assist in minimizing toxicity-related problems associated with the presence of these microbes in rough rice, thus maintaining rice quality.

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GROWTH CHARACTERISTICS OF *ASPERGILLUS FLAVUS* NRRL 3357 AND AFB1 PRODUCTION IN STORED RICE FRACTIONS

ABSTRACT

In the food industry, ensuring the microbial safety of rice is becoming increasingly important, especially when rice is used as an ingredient in ready-to-eat and minimally processed products. Mold contamination, particularly by certain *Aspergillus* species that produce toxic metabolites called aflatoxins, is a significant problem for rice, rendering it unfit for consumption by humans and animals. This study aimed to identify which storage and product-related factors significantly influence the growth of *Aspergillus flavus* NRRL 3357, ergosterol, and aflatoxin B1 (AFB1) production in different rice fractions using a three-level screening design. A follow-up experiment was then conducted to assess the impact of significant factors on AFB1 and ergosterol production at extreme levels. The studied factors included temperature, relative humidity (RH), storage duration, storage moisture content (MC), and initial *A. flavus* spore concentration. Rough, brown, and milled rice samples with different MCs (12%, 15%, and 18% w.b.) were used. The samples, both autoclaved and non-autoclaved, were inoculated with different *A. flavus* spore concentrations (1×10^4 and 1×10^6 spores/mL) and incubated at different temperatures (20°C, 25°C, and 30°C) and RHs (75%, 85%, and 95%). After incubation period of 3, 9, and 15 days, *A. flavus* plate count, ergosterol and AFB1 production were measured. The results showed that maximum *A. flavus* growth was observed at 25°C and 95% RH throughout all storage durations, as measured by plate count and ergosterol. Brown rice had the highest AFB1 concentration (9.198 μg/g) after 15 days of incubation at 30°C and 75% RH. However, during the latter part of the study, it was observed that AFB1 concentrations in brown rice inoculated with 1×10^6 spores/mL of *A. flavus* and stored at 30°C and 95% RH initially increased after 20 days and then decreased towards the end of the

storage period (60 days). These findings are expected to aid in identifying appropriate storage conditions that can reduce the risk of aflatoxin contamination and improve rice safety.

KEYWORDS.
Aflatoxin B1, *Aspergillus flavus*, Storage, Temperature, Relative humidity, Rice fractions

INTRODUCTION

One of the most serious concerns in the agricultural industry right now is the widespread contamination of various cereal grains, including rice, with aflatoxin. These toxic substances are produced as secondary metabolites by *Aspergillus* section *flavi*, comprising *Aspergillus parasiticus*, *Aspergillus flavus*, and *Aspergillus nomius* (Oduola et al., 2022). While these microorganisms primarily live in soil, they are widely distributed in nature and colonize crops to obtain organic nutrients essential for their survival and growth (Castao et al., 2017). The contamination of grains with aflatoxins can occur during crop growth as well as during harvesting, threshing, and drying, particularly through contact with infested soil. Alternatively, improper post-harvest grain storage, along with pest infestation, can easily create an environment conducive for the proliferation of aflatoxin-producing fungi, further escalating the risk of subsequent aflatoxin production (Kimatu et al., 2012).

The consumption of aflatoxin-contaminated crops can have detrimental effects on human health as well as the productivity and well-being of animals (Arapceska et al., 2015). In severe cases, it can even lead to death (Wagacha et al., 2008). These toxic compounds, classified as Group 1 carcinogens by the International Agency for Research on Cancer (IARC), are responsible for an estimated 5-28% of hepatocellular cancer cases worldwide, highlighting the substantial risk they pose to individuals (Yogendrarajah et al., 2014). Furthermore, when children are exposed to high levels of the toxin, it can result in acute poisoning, compromised immune system dysfunction, and impaired growth (Gong et al., 2016). In response to these risks, many developed countries have enacted strict regulations limiting their levels in food and feed, prohibiting the consumption of any grain exceeding specified aflatoxin thresholds (Liu et al., 2006). The European Union, for example, has established a regulatory limit of 4 µg/kg for total aflatoxins and 2 µg/kg for AFB1,

while the United States of America has set a limit of 20 μg/kg for total aflatoxins (Sales et al., 2005).

The biosynthesis of aflatoxins is influenced by a variety of factors, including nutritional, biological, and environmental factors (Abdel-Hadi et al., 2012). In some cases, they could be produced as a balancing mechanism in the fungal biological system following a period of sustained growth during which the primary metabolites required for chemical and energy processes are in excess (Daou et al., 2021). However, due to the diversity of fungal species, it is difficult to define a specific set of factors that are attributed to their production. Rather, their diversity allows them to produce toxins under a wide range of favorable environmental conditions (Liu et al., 2020). Similarly, the presence of these toxigenic species does not always imply the presence of toxins because the conditions for growth or, more specifically, toxin production differ (Mannaa et al., 2017). The conditions that promote toxin production are more stringent than those required for growth (Daou et al., 2021). Storage temperature, relative humidity, water activity, pH and storage moisture contents are among the pivotal factors that have been studied and reported to have a direct impact on aflatoxin production and fungal growth, especially during long-term storage. Additionally, the production of ergosterol, which is closely linked to active fungal biomass, can be influenced by conditions that are conducive to fungal growth (Kadakal et al., 2013 ; Bjurman et al ., 1994). Temperatures ranging from 25°C to 30°C have been found to be ideal for fungal growth, as have relative humidity levels ranging from 88% to 95% and water activity levels greater than 0.75 (Daou et al., 2021). These ideal conditions have the potential to influence the expression of the aflatoxin synthesis gene and other related regulatory factors (Wang et al., 2022).

The major chemical forms of aflatoxins that have been identified include AFB1, AFB2, AFG1, and AFG2, each with varying levels of toxicity (Castao et al., 2017). Aflatoxin B1 (AFB1)

has received the most attention due to its toxicity profile and the fact that it is almost always present in high concentrations in various agricultural crops consumed by humans, such as corn, rice, wheat, peanuts, dried fruits, sorghum, and others (Oduola et al., 2022). Remarkably, the distribution and concentrations of AFB1 can vary among different fractions of rice due to the inherent physical structure of the grains. While previous findings have shown that removing the bran during milling significantly reduces AFB1 levels, nevertheless it is critical that all fractions are stored under suitable conditions that may not accelerate toxin production (Castells et al., 2007; Schroeder et al., 1968; Liu et al., 2006). Furthermore, it is essential to recognize that aflatoxin contamination is an accumulative process that can initiate on the farm and can increase along any stage along the processing chain (Norlia et al., 2019).

Although rice is less susceptible to AFB1 contamination compared to other cereal grains, there is still a considerable risk of exposure in countries where rice is the primary staple food source. For instance, an adult in Pakistan consumes about 171 g of rice per day, when considering toxicological references expressed per body weight, the estimated risk assessment for dietary exposure to aflatoxins ranges from 19.1 to 26.6 ng/kg body weight per day (Iqbal et al., 2016). Besides, ensuring the safety of rice is crucial, as it is a vital crop that constitutes a significant portion of people's daily caloric intake worldwide (Romero-Sánchez et al., 2022). Aside from aflatoxin production, fungal contaminants cause organoleptic changes such as grain discoloration and mustiness, resulting in significant economic losses for the food industry (Wang et al., 2022). Each year, about 15% of cultivated rice is discarded due to fungal contamination and presence of other harmful species (FAO) (Hassan et al., 2022).

Despite numerous attempts utilizing biological, chemical, and physical methods, the elimination of aflatoxins has proven to be ineffective, primarily due to their resistance and

persistence. These toxins remain present in crops even after undergoing high-temperature processing, such as heating at 150-200°C (Oduola et al., 2022). Moreover, it is challenging to eliminate them entirely while maintaining the sensory and nutritional quality of the grain. With this in mind, to effectively address aflatoxin formation, one of the key strategies is the early inactivation of these toxigenic species upon detection (Oduola et al., 2022), coupled with identifying the appropriate storage conditions that can suppress their growth (Asghar et al., 2014). Extensive research underway aims to develop strategies that can effectively combat aflatoxin contamination, with a particular emphasis on identifying safe storage conditions.

Several other studies have also implemented mathematical models to assess the impact of factors like temperature and water activity on fungal behaviors (Phan et al., 2022; Garcia et al., 2022). Nevertheless, very few studies have been conducted to investigate aflatoxin B1 (AFB1) contamination in various rice fractions. Hence, the objectives of this study were to (1) identify which storage and product-related factors significantly influence the growth of *Aspergillus flavus*, ergosterol, and aflatoxin B1 (AFB1) production in different rice fractions using a three-level screening design and (2) elucidate the degree of influence of significant factors at extreme levels. The examined factors included storage temperature, relative humidity (RH), storage duration, rice moisture content (MC), type of rice fraction (rough, brown, and milled rice), and initial *A. flavus* spore concentration.

MATERIALS AND METHODS

SAMPLE PROCUREMENT AND PREPARATION

Freshly harvested long-grain rough rice (RT 7521) with a moisture content of about 18.3% (w.b.) procured from a farm in Hazen, Arkansas, was used in this study. The rice was cleaned upon

delivery using dockage equipment (Mci kicker dockage tester, mid-continent industries inc., Newton, KS) and then divided into three batches (Batch 1, 2 and 3), each weighing about 2.4 kg. In an equilibrium moisture content (EMC) controlled chamber at 25°C and 56% RH, batch 1 was dried to three different moisture contents: 18%, 15%, and 12% moisture content (w.b.), while batch 2 and 3 were gently dried to 12% moisture content (w.b.). The moisture content of the samples was determined using an AM 5200 grain moisture tester (Perten instruments, Hägersten, Sweden). To obtain brown rice, batch 2 and 3 (12% moisture content w.b.) were dehulled using a huller (THU-411 35A, Satake engineering, Tokyo, Japan). Then, one batch was milled for 27 seconds in a laboratory mill (McGill 412 number 2, Rapsco, Brookshire, TX, USA) to achieve a standardized surface lipid content (SLC) of 0.4%. The head rice for both brown and milled rice was separated from the broken rice using a grain separating device (Grain machinery 414 manufacturing, Miami, FL, USA). The head rice was any kernel that was at least three-quarters the length of a whole kernel. Following that, all samples from all three batches were transferred to sterile jars, and approximately 1.2 kg of samples from each batch were autoclaved, excluding the other half. Prior to any further experiments, both the autoclaved and non-autoclaved samples were stored in a laboratory cold room set at 4°C for 48 hrs.

SURFACE LIPID CONTENT DETERMINATION

The surface lipid content of the milled rice was determined using a near infrared reflectance (NIR, DA7200, Perten instrument, Hagersten, Sweden). Approximately about 50 g of the milled rice was filled inside the equipment cup then placed under the equipment's focusing area where the infrared beam could easily project on it. The algorithm provided by the manufacturer of the equipment converts the sensors data to the SLC data through calibration (Matsler and Siebenmorgen, 2005; Saleh et al., 2008; Olatunde and Atungulu, 2018).

ASPERGILLUS FLAVUS SPORE PREPARATION

Toxigenic A. *flavus* strain NRRL 3357 was obtained from a glycerol stock that had been stored in a -80°C freezer in Dr. Burton Bluhm's laboratory at the University of Arkansas, Fayetteville, USA. After that, the mold was then grown on potato dextrose agar (PDA) and incubated at 25°C. Sub-culturing was performed on a fresh Potato Dextrose Agar plate. *A. flavus* spores were obtained after 7 days of incubation by flooding the plates with sterile distilled water and gently scraping with a sterile L-shaped plastic spreader. Then, the spore concentration was determined using a hemocytometer and a microscope. The spore concentrations were adjusted using a microscope to 10^4 and 10^6 spores/mL, respectively, using an appropriate dilution.

INOCULATION OF RICE SAMPLES

Afterwards, 50 g of rough, brown, and milled rice samples (autoclaved and non-autoclaved) were placed in separate 250 mL sterile flasks and inoculated with 1×10^4 and 1×10^6 spores/mL of *A. flavus*. Control samples were not inoculated. After inoculation, the flasks containing the samples were shaken for about 2 minutes to ensure consistent mixing of the inoculum with the rice kernels. The samples were then carefully transferred into sterile square cut cheese cloths, followed by bringing the four corners together and tied securely with a sterile string. The wrapped cheesecloths containing the rice samples were then suspended in jars filled with saturated salt solutions, sealed, and incubated for 3, 9, and 15 days, respectively, at the designated experimental temperatures of 20°C, 25°C, and 30°C. The use of saturated salt solutions allowed for the creation of controlled environmental chambers, providing specific relative humidity levels of 75%, 85%, and 95% for the duration of the study. In the subsequent phase of the study, which served as a follow-up experiment to the screening study, 50 g of rough and brown rice were inoculated with a concentration of 1×10^6 spores/mL of *A. flavus*. The storage duration was

extended to 20, 40, and 60 days, with temperatures set at 20°C and 30°C, while maintaining a constant relative humidity of 95%.

PLATE COUNT

A sterile stomacher bag was filled with 10 g of rice. Then, 90 mL of sterile phosphate-buffered solution (PBS) dilution water was added, carefully sealed, and placed in a stomacher blender, where it was agitated for 4 minutes at 260 rpm. Serial dilutions were performed to obtain different dilution levels by combining 1 mL of the original mixture in the stomacher bag (first dilution 10^{-1}) with 9 mL of sterile PBS dilution water in a test tube (second dilution 10^{-2}). This process was repeated for subsequent dilutions until reaching the seventh dilution (10^{-7}). Potato Dextrose Agar plates (PDA) containing streptomycin and chlortetracycline as antibiotics were used to count the colonies of *A. flavus* and other natural fungi in both autoclaved and non-autoclaved rice samples. A 0.1 mL sample solution from each dilution was transferred to the center region of the PDA plate. The sample was then spread evenly with a sterile plastic spreader (L-shaped).The inoculated PDA plates were then incubated at a temperature of 25°C for 72 hours to allow the colonies to grow. After the incubation period, the number of colonies (CFU/g) was counted to assess the fungal population in the rice samples.

The colony-forming unit per gram of rice (CFU/g) for each sample was obtained using equation 1.

$$T_{cfu} = \frac{P_{cfu}}{D_r}$$

where

T_{cfu} = total colony forming units per gram of rice (CFU/g),

P_{cfu} = colony forming units counted on the plate per gram of rough rice, and

D_r = dilution rate 10^{-1} to 10^{-6} durations.

CHEMICALS AND EQUIPMENT

The chemicals used in this study, including hexane, methanol, and potassium hydroxide (KOH), as well as the ergosterol and AFB1 standards, were obtained from Sigma Aldrich and VWR (USA). HPLC grade hexane and methanol with a purity of 99.9% were used. Ultrapure water from Barnstead, Smart2pure 6 UV/UF (Langenselbold, Germany), was utilized for the extraction and HPLC analysis. The ultrapure water was further filtered using a 0.45-μm filter.The HPLC system employed in this study was a Shimadzu 20A series HPLC system from Tokyo, Japan. The system consisted of an autosampler (SIL-20AHT), a pump (LC-20AT), a low-pressure gradient pump unit (LC-20AD/T LPGE kit), a degasser (DGU-20A5), a photodiode array detector (SPD-M20A), a fluorescence detector (RF-10AXL), a column oven (CTO-20A), a photochemical reactor for enhanced detection (PHRED), an analytical column - Luna 5μ C18 100A; 250 × 4.60 mm (Phenomenex), a guard column, and mobile phases A (ultrapure water) and B (methanol).

EXTRACTION, MEASUREMENT AND QUANTIFICATION OF AFB1 AND ERGOSTEROL

ERGOSTEROL EXTRACTION

The method used for extracting ergosterol, with slight modifications, followed the procedure described by Femenias et al. (2021). In a 25 mL scintillation vial, 8 mL of methanol and 2 mL of hexane were measured. Then, 2 g of homogenized rice samples were carefully weighed and added to the scintillation vial. The vial was vortexed for 2 minutes and placed on an orbital shaker set to 180 rpm for 30 minutes to ensure thorough extraction. After allowing the mixture to settle, 6 mL of the supernatant (methanol and hexane) was transferred to a 15 mL centrifuge tube. To this, 0.6 g of KOH was added, and the contents were dissolved. The centrifuge tube was then placed in a hot water bath set at 55°C. After 20 minutes, the tube was removed and allowed to cool

to room temperature (around 23°C). The tube was then agitated by vortexing and shaking with hands after 0.8 mL of ultrapure water was added. The upper layer (hexane) was transferred into a glass test tube using a Pasteur pipet after the hexane-methanol mixture separated. The hexane extraction was repeated twice with 1 mL of hexane each time. The extracted hexane was then combined and dried under a gentle stream of nitrogen. After drying off the hexane, the extract was resuspended in 1 mL of methanol and filtered into an HPLC vial using a 0.22 μm nylon syringe filter. After allowing the mixture to settle, 6 mL of the supernatant (methanol and hexane) was transferred to a 15 mL centrifuge tube. To this, 0.6 g of KOH was added, and the contents were dissolved. The centrifuge tube was then placed in a hot water bath set at 55°C. After 20 minutes, the tube was removed and allowed to cool to room temperature (around 23°C).

The tube was further agitated by vortexing and shaking manually after the addition of 0.8 mL of ultrapure water. Following the separation of the hexane and methanol layers, the upper layer (hexane) was transferred into a glass test tube using a Pasteur pipet. The hexane extraction was repeated twice, each time with 1 mL of hexane. The extracted hexane was combined and dried under a gentle stream of nitrogen. Once the hexane was dried off, the extract was resuspended in 1 mL of methanol and filtered into an HPLC vial using a 0.22 μm nylon syringe filter.

ERGOSTEROL MEASUREMENT

The method described by Waskiewicz et al. (2010) was used with a minor modification. Ergosterol was measured using an isocratic mobile phase (methanol) at a flow rate of 1.0 mL/min. The HPLC instrument was injected with 20 μL of ergosterol, and the column temperature was set to 25°C. The UV wavelength of the photodiode array detector was set to 282 nm. Ergosterol was detected at approximately 13.3 minutes and quantified by comparing the peak areas to those of an

external standard. The calibration curve with a R square of 0.998 was constructed using five concentrations of the ergosterol standard (1, 50, 100, 250, and 500 μg/g).

AFLATOXIN EXTRACTION

AFB1 extraction was performed using a slightly modified protocol described by Kamala et al. (2016). A 25 mL scintillation vial was filled with 12 mL of 80% methanol (80:20, methanol:water v/v). Following that, 2 g of the homogenized rice sample was carefully weighed into a scintillation vial containing 80% methanol and then vortexed for 2 minutes to ensure even mixing. The scintillation vial was then placed on an orbital shaker set to 180 rpm for 16 hours (or overnight). After 16 hours of mixing, 1.5 mL of the sample was filtered into a 2 mL Eppendorf tube using Whatman filter paper No 4. Following that, 1 mL of the filtrate was passed through a 0.22 μm nylon syringe filter and into an HPLC vial. After that, the HPLC vial was loaded into the HPLC instrument for analysis. If the extracts were not analyzed right away, they were kept in a -20°C freezer to avoid degradation.

AFLATOXIN MEASUREMENT

The method used in this study was slightly modified from Waltking, Wilson, and Dunn (2006). Gradient program for mobile phase (0.01-2 minutes: 70% mobile phase A - 30% mobile phase B; 2.01-9.00 minutes: 40% mobile phase A - 60% mobile phase B; 9.01-12.00 minutes: 70% mobile phase A - 30% mobile phase B). The injection volume was 10 μL and the flow rate was set to 1.0 mL/min. The column temperature was set to 30°C, and the fluorescence detector equipped with a post-column photochemical derivatization was set to 365 nm (excitation wavelength) and 435 nm (detection wavelength) (emission wavelength). At 9.4 minutes, AFBI was detected. AFB1

was quantified using a calibration curve with known standard concentrations (0.005-1.5 μg/g) and a R square of 0.998.

EXPERIMENTAL DESIGN

This study utilized a discrete screening design (DSD), a statistical tool used to identify the most important factors and any interaction effects among many potential factors. It uses a three-level design to evaluate significant factors and help eliminate those that have no significant effect on the response variable (Yan et al., 2021). The design utilizes a sensible range for the three levels, which correspond to the minimum, middle, and maximum levels of a factor. A subsequent run can then be performed to determine the nature of the interaction among the significant factors (Yan et al., 2021; Montgomery, D.C., 2017). In this case, the DSD design was used to identify the factors that significantly influenced aflatoxin (AFB1) production and fungal population (using plate count and ergosterol) in different rice fractions (rough, brown, and milled rice). Tables 1 and 2 show the three-level structure of the DSD design with the factors. Levels 1, 2, and 3 are denoted by -1, 0, and 1, respectively, and correspond to the minimum, middle, and maximum settings. The columns of the design indicate that the factors are orthogonal, which implies that the factors are statistically independent of each other. The six studied factors included storage temperature (20°C, 25°C, 30°C), storage relative humidity (75%, 85%, 95% RH), storage duration (3, 9, 15 days), rice moisture content (12%, 15%, 18% MC), autoclave (yes or no), and the *A. flavus* spore concentration (10^4, 10^6, control)

Table 1: Factors at three level by discrete screening design

Factors	Levels		
	Minimum (-)	**Middle (0)**	**Maximum (+)**
a - storage MC (%)	12	15	18
b - mold load (spores/mL)	control	10^4	10^6
c - storage temperature (°C)	20	25	30
d - storage RH (%)	75	85	95
e - storage duration (Day)	3	9	15

Table 2: Experimental design of the six factor combinations using a DSD

Run	Autoclave	Run sequence	Factors				
			a	b	c	d	e
1	Yes	--++0	12	control	30	95	9
2	Yes	-0--+	12	10^4	20	75	15
3	Yes	-+-++	12	10^6	20	95	15
4	Yes	-++0-	12	10^6	30	85	3
5	Yes	0----	15	control	20	75	3
6	Yes	00000	15	10^4	25	85	9
7	Yes	+-0++	18	control	25	95	15
8	Yes	++-+-	18	10^6	20	95	3
9	Yes	+++-+	18	10^6	30	75	15
10	No	---+-	12	control	20	95	3
11	No	--+-+	12	control	30	75	15
12	No	-+0--	12	10^6	25	75	3
13	No	00000	15	10^4	25	85	9
14	No	0++++	15	10^6	30	95	15
15	No	+--0+	18	control	20	85	15
16	No	+-+--	18	control	30	75	3
17	No	+0++-	18	10^4	30	95	3
18	No	++--0	18	10^6	20	75	9

STATISTICAL ANALYSES

A statistical software package (JMP version 16.0.0, SAS Institute) was used to generate and analyze the DSD, with 18 runs repeated for each rice fraction (rough, brown, and milled rice) and a completely randomized factorial design for the follow-up part of the study, with runs for all treatment combinations (rough and brown rice). Analysis of variance (ANOVA) and Tukey's

honest significance difference test were used to determine the significant differences among the samples. The model's coefficient was fitted using regression analysis.

RESULTS AND DISCUSSION

In the early stages of this study, the primary goal of the DSD (Discrete screening design) was to help identify the most important factors that affect the growth kinetics of *A. flavus*, ergosterol and aflatoxin B1 (AFB1) production from among numerous potential factors.

SCREENING ANALYSES OF *A. FLAVUS* (3357) GROWTH, ERGOSTEROL AND AFB1 PRODUCTION IN RICE FRACTIONS

For a long time, traditional plate counts have been used to identify and quantify mold populations in food. However, researchers have discovered that ergosterol, a specific component found in the cellular walls of fungi, can be used effectively for early detection of fungi (Martin et al., 1990; Saxena et al., 2001; Seitz et al., 1979; Tothill et al., 1992; Naewbanij et al., 1984). In the same way, storage conditions that hinder fungal growth are likely to result in a decrease in ergosterol levels (Mille-Lindblom et al., 2004). Previous studies have shown that estimating fungal biomass using ergosterol is more reliable because it can detect non-viable forms of mold that do not grow on culture media but are still capable of producing toxin (Saxena et al., 2001). Moreover, ergosterol results can be obtained more quickly compared to the plate count technique, which requires an incubation period. In addition, the plate count technique may have wide variability with sample homogenization in terms of stomacher blending, which may only enumerate fungi based on surface colonization. In the present study, we examined the impact of storage parameters on the growth kinetics of *A. flavus* in rough, brown, and milled rice, based on ergosterol concentration and plate count.

Table 3 shows the factors examined that affected *A. flavus* growth in different rice fractions (rough, brown, and milled), along with their corresponding p-values. A moderate correlation (r = 0.555) between ergosterol production and plate count was observed, as depicted in figure 1. The storage conditions that resulted in the highest mean ergosterol concentrations, as observed in figures 2 and 3, were found to be similar to those associated with the highest mean plate counts. These conditions included temperatures of 25°C and 30°C, as well as relative humidity (RH) values of 85% and 95%. These findings were not surprising given that plate count and ergosterol are both used to measure fungal growth. Oduola et al. (2022) also found a strong correlation (r = 0.797) between plate count and ergosterol levels in *A. flavus*-inoculated corn. The slight discrepancy may be due to differences in food substrates and the standardized conditions of their study, whereas our study included a variety of storage conditions before measuring ergosterol and plate count. Furthermore, Cahagnier et al. (1993) observed that ergosterol biosynthesis was more favorable in corn compared to rice. Clearly, if the conditions are not standard, this relationship can become complex (Gourama et al., 1995).

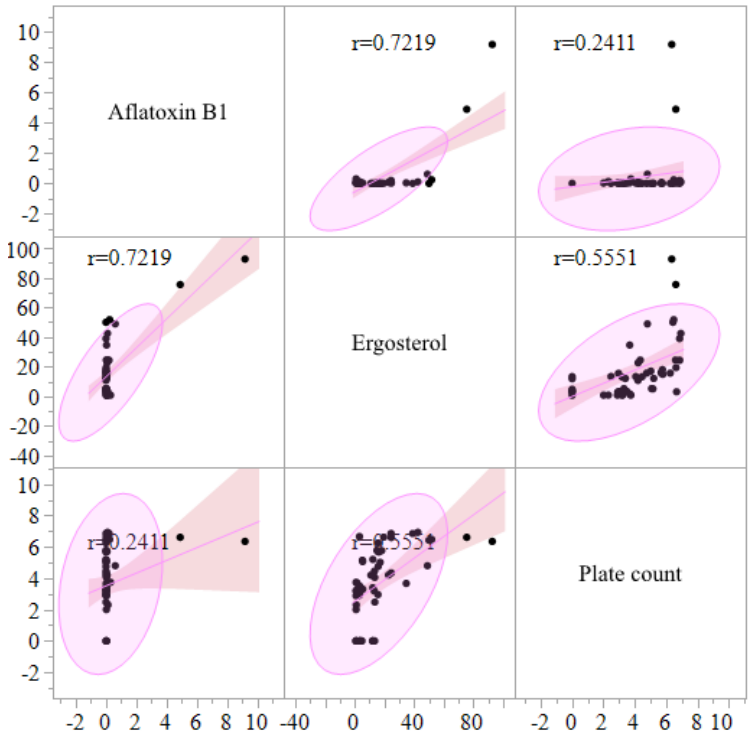

Fig 1: A scatter plot matrix showing the correlation between Aflatoxin B1, ergosterol and plate count

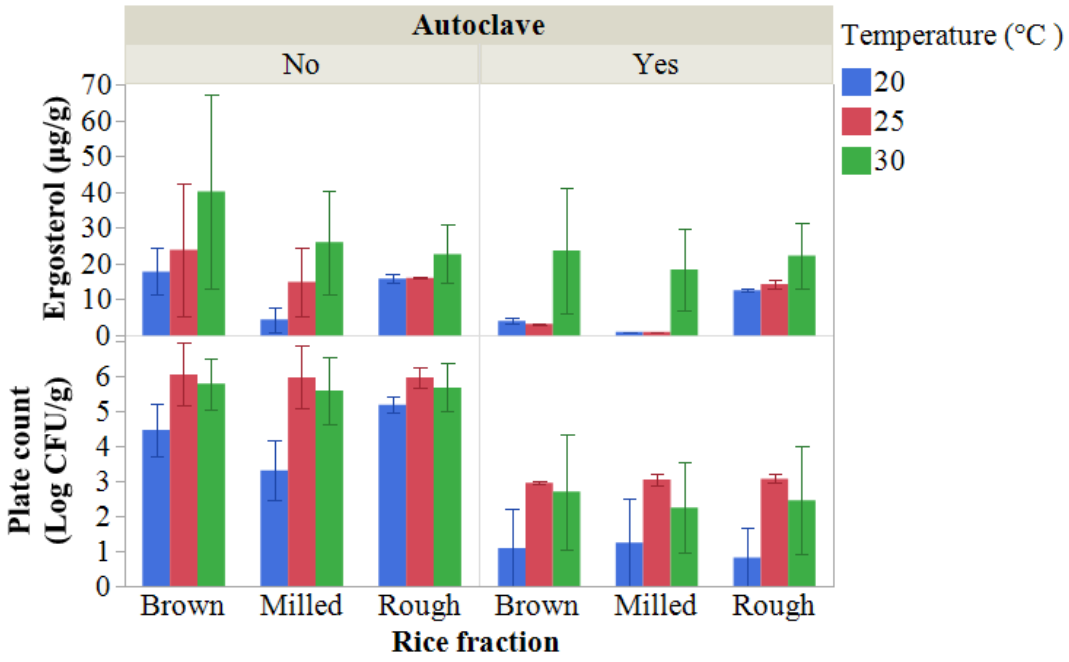

Fig 2: Effect of temperature on mean plate count and ergosterol in brown, milled and rough rice

Table 3: Factors influencing the growth kinetics of *A. flavus* and potential *A. flavus* in rough, milled and brown rice, respectively with their corresponding p values

Factor	Rough rice (Prob> |t|)	Brown rice (Prob> |t|)	Milled rice (Prob> |t|)
Autoclave (no)	<.0001*	0.0008*	0.0096*
Temperature	0.0054*	0.0253*	0.2027
RH	0.0090*	0.0420*	0.2623
Mold load	0.0198*	0.0118*	0.0329*
Storage duration	0.9840	0.0949	0.1826
Temp*RH	0.0038*	0.0161*	0.1419

[1] Storage factors with corresponding p values for each rice fraction, including rough, brown, and milled rice, are listed in rows. The p value determines the effect of each factor on growth of *A. flavus* in the rice samples (P >0.05 not significant)

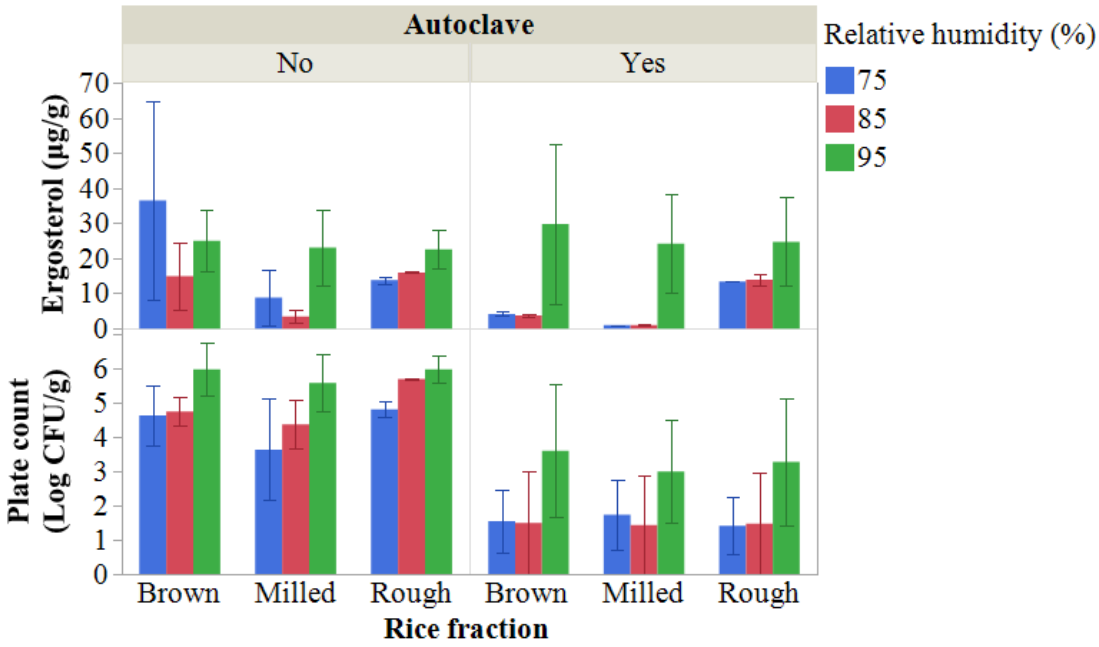

Fig 3: Effect of relative humidity on mean plate count and ergosterol in brown, milled and rough rice

1

The initial mean fungal populations in the non-autoclaved (RT7521) rough, brown, and milled rice were 5.18 log CFU/g, 4.81 log CFU/g, and 4.42 log CFU/g, respectively. However, it is important to note that our study did not conduct any qualitative analyses to specifically identify the fungal isolates naturally present in the non-autoclaved rice samples. Therefore, the fungal populations were reported as fungal plate counts. Nonetheless, previous research by Castao et al. (2017) revealed the presence of various predominant fungal species such as *Aspergillus*, *Alternaria*, *Penicillium*, *Rhizopus*, *Fusarium*, *Phoma* and others in paddy and brown rice. Similarly, Schroeder et al. (1973) found that approximately one-third of the *A. flavus* isolates detected in rice grown in the United States were capable of producing significant amounts of aflatoxins.

The growth kinetics of *A. flavus* differed significantly between autoclaved and non-autoclaved rice fractions ($p\ value < 0.05$). The inclusion of both autoclaved and non-autoclaved samples in the screening study aimed to explore the potential influence of existing fungal communities on the growth of *A. flavus* and the production of aflatoxin B1 (AFB1). Clearly, as anticipated the existing microflora in non-autoclaved rice samples likely hindered the growth of *A. flavus* by competing for nutrients and resources, thereby limiting their growth. It is also possible that the interactions between the different fungal communities could have induced changes in the environmental conditions, such as pH or nutrient availability, which may have impacted the growth and toxin production of *A. flavus*. In a study by Abdelaziz et al. (2022), it was found that *A. fumigatus* exerted antifungal properties, inhibiting approximately 77% of *A. flavus* growth and toxin production.

The growth of *A. flavus* in rough and brown rice was significantly influenced by temperature and relative humidity (RH). Moreover, there was an interaction effect between

temperature and RH, indicating that the impact of temperature on fungal growth depended on the level of RH within the storage environment. Lower RH levels at a constant temperature led to decreased fungal growth, while higher RH levels at a constant temperature were associated with increased fungal growth. These findings align with the research conducted by Chai et al. (2019), who also observed that the interaction effects of temperature and RH had a more substantial influence on fungal composition compared to RH alone.

In addition to temperature and relative humidity (RH), the growth of *A. flavus* was also influenced by the type of rice fraction it grew on. The highest plate counts were observed in milled and brown rice when stored at 25°C and 95% RH, resulting in an increase of 3.41 log CFU/g and 2.12 log CFU/g, respectively, after 15 days from their initial counts (Figure 5). On the other hand, the most favorable growth conditions for rough rice were observed at 30°C and 95% RH, leading to an increase of 1.67 log CFU/g (Table 4). The intact hull in rough rice may have limited *A. flavus* from accessing the starchy endosperm for energy, necessitating higher temperatures to accelerate the chemical reactions required for fungal growth (Daou et al., 2021).

Conversely, under suboptimal conditions (30°C and 75%), the growth of *A. flavus* depended on the availability of nutrients in the substrate (Marin et al., 1999). When the rice samples were stored at 30°C and 75% RH, fungal growth increased specifically in brown and milled rice, while a significant reduction was observed in rough rice (Figure 4). In fact, at the lowest temperature limit of 20°C and 75% RH, growth decreased in all rice fractions (Fig 4). Although fungi typically metabolize glucose for growth, they are also able to hydrolyze the available lipids in brown rice using metabolic enzymes (Juntachai et al., 2009).

The nutrient-rich composition of milled and brown rice provided adequate food source for *A. flavus*, resulting in enhanced fungal growth. Dharmaputra (1997) discovered a positive

relationship between surface lipid content and total fungal population, indicating that the higher the surface lipid content, the greater the fungal growth.

Interestingly, the growth pattern of *A. flavus* in autoclaved rice samples differed from that of non-autoclaved rice samples. The highest plate counts of *A. flavus* were observed in brown rice, followed by rough rice, and then milled rice when stored at 30°C and 95% RH for 15 days (Figure 7), resulting in approximately 6.61 log CFU/g, 6.43 log CFU/g, and 4.80 log CFU/g for brown, rough, and milled rice, respectively. The most probable reason could be that since autoclaving involved subjecting rice samples to high temperatures and pressure, that may have weakened the structural integrity of the hull in rough rice kernels, creating openings that facilitated the penetration and colonization of *A. flavus*. Phan et al. (2022) also reported a similar trend, observing a maximal growth rate in paddy rice (rough rice) compared to milled rice at 30°C and 0.95 water activity. On the other hand, at 25°C and 85% RH, there was no significant difference in fungal populations between autoclaved and non-autoclaved rice fractions (Figure 6 and 9

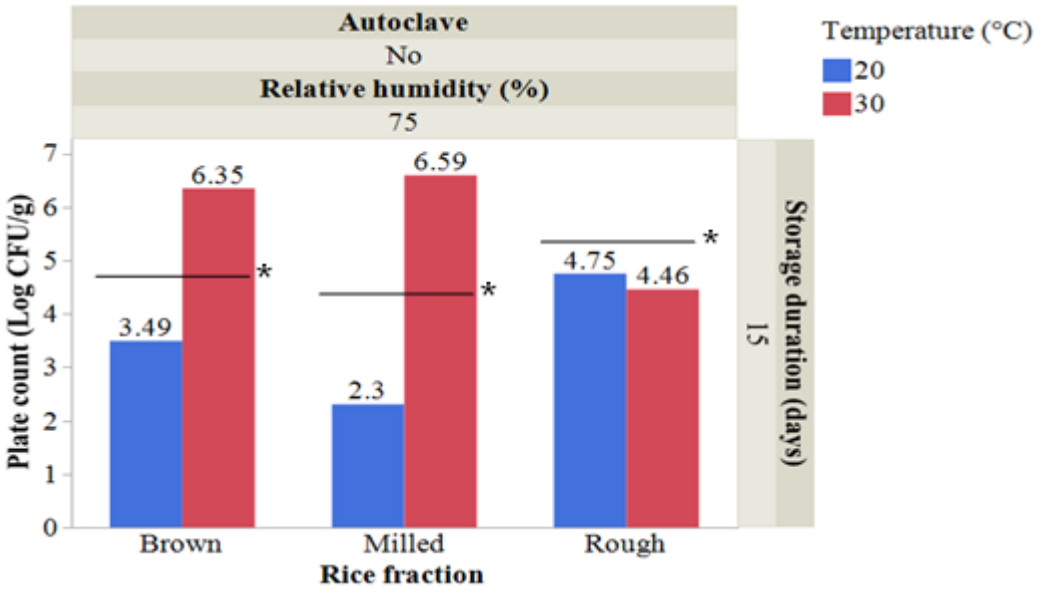

Fig 4: Effect of temperature and relative humidity interaction on plate count in brown, milled and rough rice after 15-day storage period. The rice samples (non-autoclaved) were stored at (20°C and 75%) and (30°C and 75%) *The line signifies initial fungal populations, 5.18 log CFU/g, 4.81 log CFU/g, and 4.42 log CFU/g for rough, brown, and milled rice, respectively.

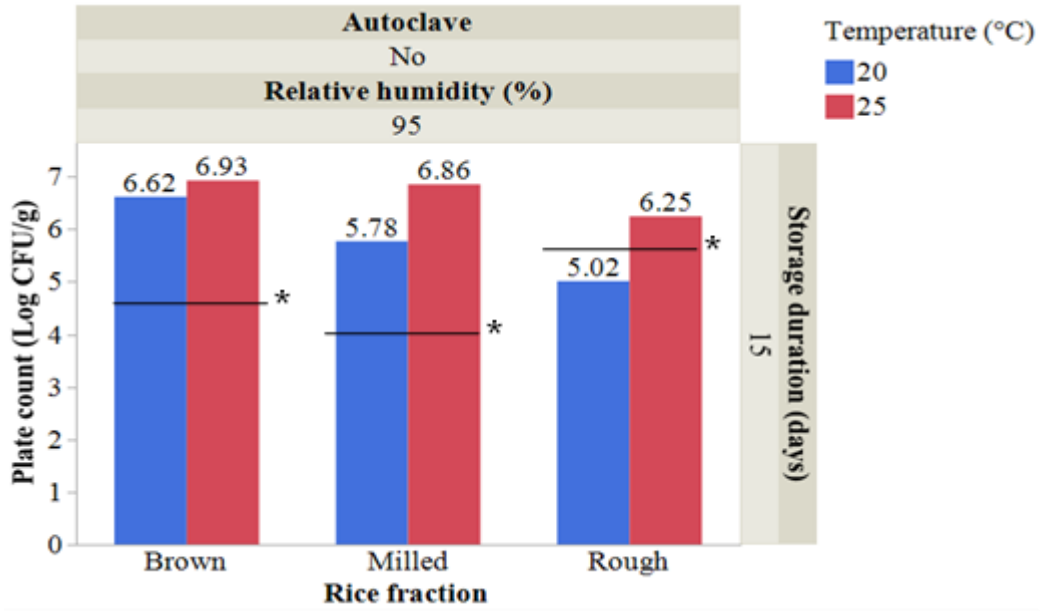

Fig 5: Effect of temperature and relative humidity interaction on plate count in brown, milled and rough rice after 15-day storage period. The rice samples (non-autoclaved) were stored at (20°C and 95%) and (25°C and 95%) *The line signifies initial fungal populations, 5.18 log CFU/g, 4.81 log CFU/g, and 4.42 log CFU/g for rough, brown, and milled rice, respectively

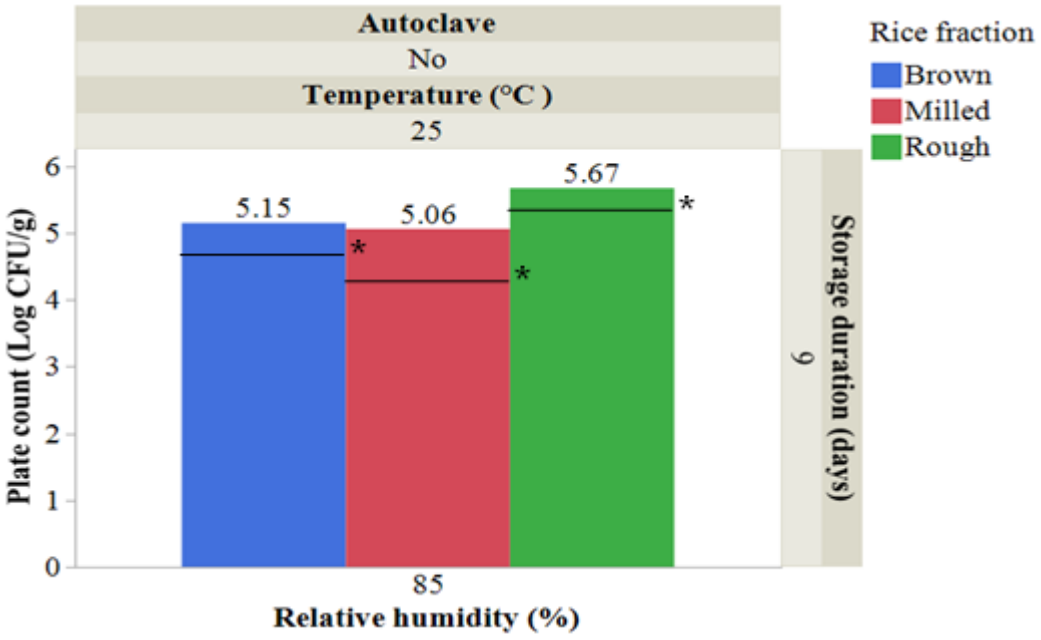

Fig 6: Effect of temperature and relative humidity interaction on plate count in brown, milled and rough rice after 9-day storage period. The rice samples (non-autoclaved) were stored at 25°C and 85% RH. *The line signifies initial fungal populations, 5.18 log CFU/g, 4.81 log CFU/g, and 4.42 log CFU/g for rough, brown, and milled rice, respectively.

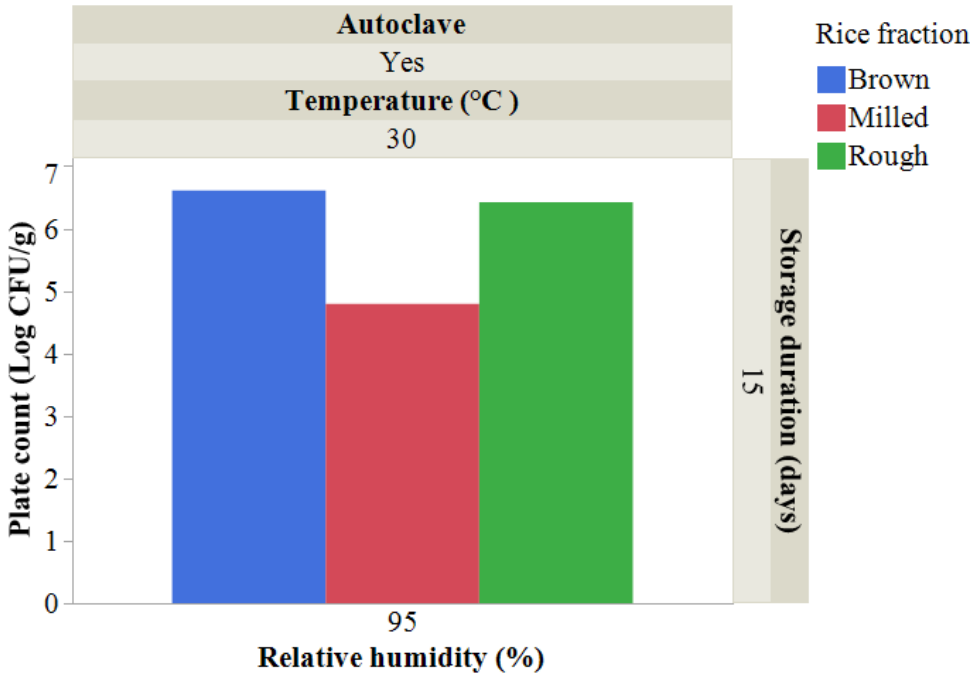

Fig 7: Effect of temperature and relative humidity interaction on plate count in brown, milled and rough rice after 15-day storage period. The rice samples (autoclaved) were stored at 30°C and 95% RH.

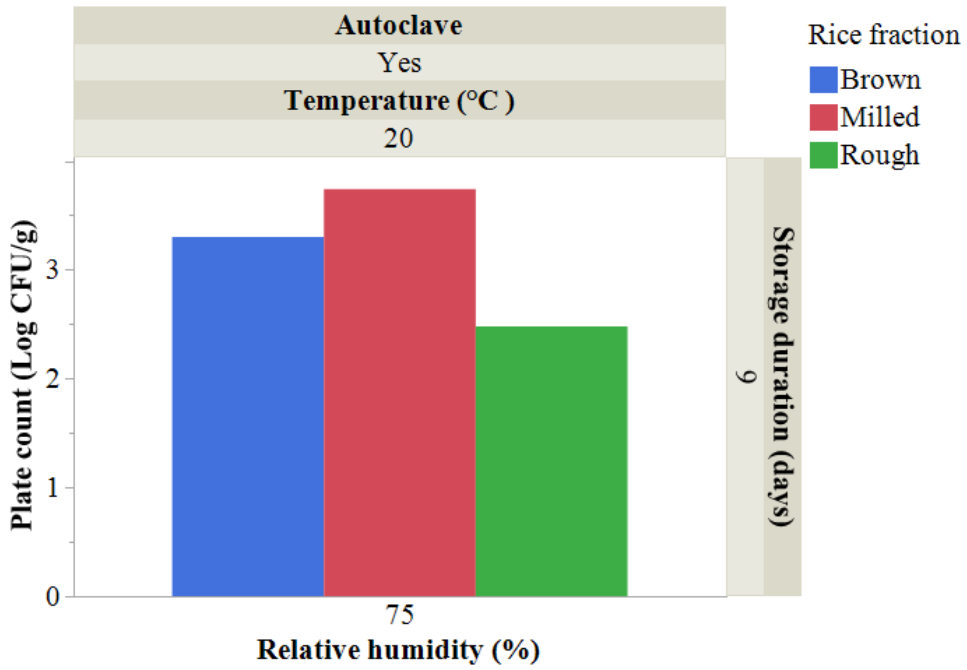

Fig 8: Effect of temperature and relative humidity interaction on plate count in brown, milled and rough rice after 9-day storage period. The rice samples (autoclaved) were stored at 20°C and 75% RH.

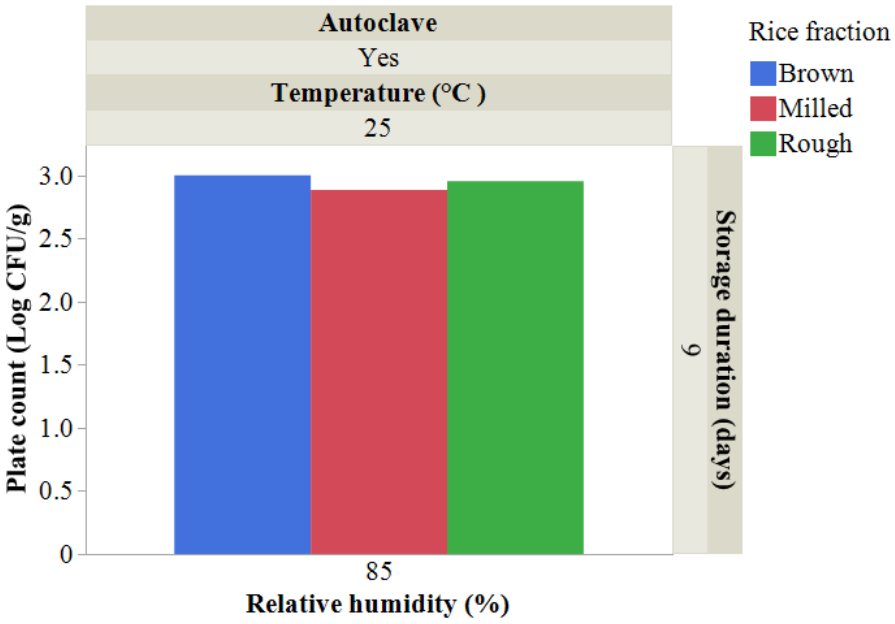

Fig 9: Effect of temperature and relative humidity interaction on plate count in brown, milled and rough rice after 9-day storage period. The rice samples (autoclaved) were stored at 25°C and 85% RH.

SCREENING ANALYSES OF AFB1 PRODUCTION IN RICE FRACTIONS

Temperature had the most profound influence on AFB1 production in rough and brown rice. Previous studies have reported that temperatures between 25°C and 30°C are ideal for AFB1 production by *A. flavus* (Kheiralla et al., 1992; Giorni et al., 2007; Takahashi et al., 1989). In this study, AFB1 production was predominantly observed in rice samples stored at 30°C and 95% RH (Fig 10). However, determining optimal conditions for AFB1 production can be challenging due to the impact of other factors such as substrate type and fungal strain occurring naturally in the crops (Klich, 2007). Lv et al. (2019) found that temperature, along with water activity, relative humidity, and substrate type, influenced the expression of genes involved in the biosynthetic pathway of AFB1.

Notably, AFB1 production was observed in brown and milled rice over a wider range of storage parameters, as shown in tables 5 and 6, respectively. Particularly, brown rice (non-autoclaved) had the highest AFB1 concentration (9.198 µg/g) after 15 days of incubation at 30°C and 75% RH (Fig 10). These findings align with a study by Takahashi et al. (1989), where substantially higher amounts of AFB1 were observed in inoculated brown rice stored at 28°C and 85-95% RH for 15 days compared to milled rice. Similarly, Sales and Yoshizawa (2005) reported considerably higher concentrations of AFB1 in naturally contaminated brown rice (2.7 µg/kg) compared to milled rice (0.37 µg/kg). The presence of lipids in brown rice bran not only serves as a preferred carbon source influencing fungal growth but also greatly induces aflatoxin production, as acetyl CoA derived from lipid ß-oxidation serves as an essential precursor in the aflatoxin biosynthesis pathway (Maggio-Hall et al., 2005). Furthermore, the low relative humidity (75%) may have subjected *A. flavus* to moisture stress, triggering physiological and biochemical signals that lead to increased AFB1 production. Abiotic stressors and other stressors, such as fungicides, as reported by Schmidt-Heydt et al. (2008), can influence the expression of biosynthesis genes

involved in mycotoxin production (Doohan et al., 1999). It is also possible to speculate that *A. flavus* produced higher toxin levels as a survival strategy, potentially driven by its attempt to outcompete existing microflora for available resources under adverse conditions. As a xerotelant microorganism, *A. flavus* can withstand low water conditions, giving it a competitive advantage over other fungal communities that are unable to thrive under adverse conditions (Klich 2007). In milled rice, the duration of storage had a significant impact on AFB1 production. The highest concentration (0.612 μg/g) was observed after 15 days of storage at 30°C and 95% relative humidity (RH), as indicated in Table 6. Prolonged storage of milled rice under conditions that promote AFB1 production increases the risk of contamination.

However, very low concentrations of AFB1 were detected in rough rice (0.066 μg/g and 0.03 μg/g) during incubation at 30°C with RHs of 85% and 95%, respectively as shown in Table 4. Interestingly, the storage moisture content did not have a significant impact on AFB1 production and *A. flavus* growth ($p = 0.9897$) in rough rice. These findings are consistent with the results reported by Muga et al. (2019), where moisture content was found to have no significant effect on aflatoxin contamination in corn. This can be attributed to the Equilibrium Moisture Content (EMC) phenomena of the grain, which relies on both temperature and relative humidity (Cotty & Jaime-Garcia, 2007). Since these two parameters are interconnected, the moisture content and water activity of the grain fluctuate as the grain either loses or absorbs moisture until it reaches equilibrium with the ambient air. When the relative humidity is high in the storage environment, moisture is absorbed back into the grain, and vice versa (Kaaya & Kyamuhangire, 2006). Furthermore, regardless of moisture content, temperature, RH, or storage duration, no detectable AFB1 concentrations were observed in any of the other rough rice samples (Table 4). These results suggest that brown rice and milled rice may be more susceptible to AFB1 contamination than

rough rice. This could be attributed to the presence of the hull in rough rice, which acts as a protective barrier against *A. flavus* invasion. Once the hull is removed from rough rice, the fungi can more easily access the bran layer and starchy endosperm.

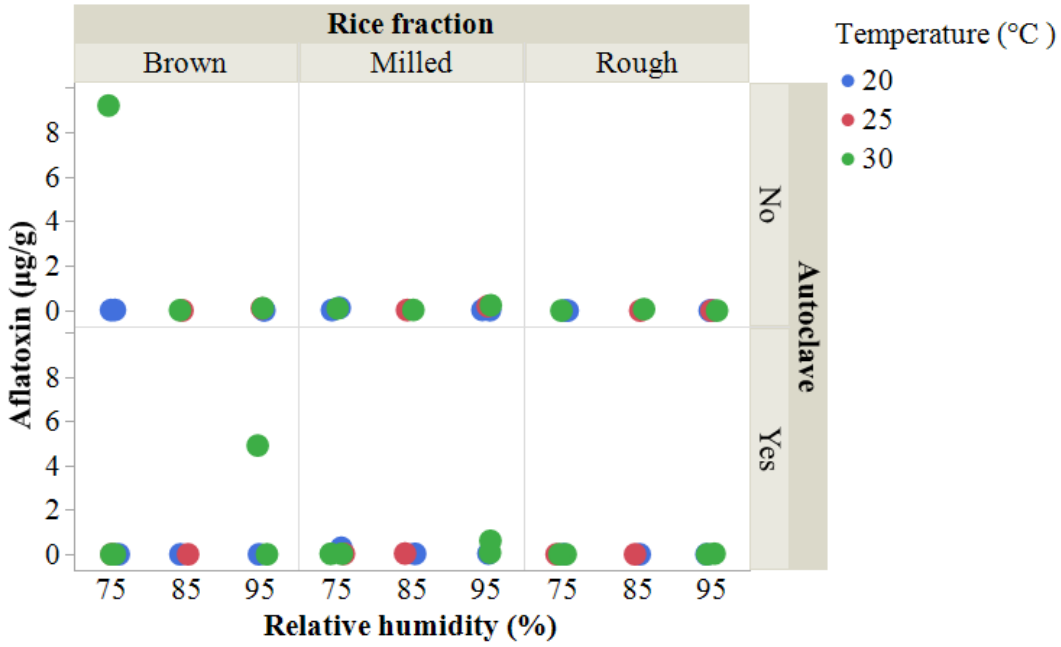

Figure 10. Aflatoxin B1 (AFB1) concentration in brown, milled and rough rice as an effect of temperature and relative humidity. No (non-autoclaved rice samples), Yes (autoclaved rice samples).

Table 4: The concentrations of Aflatoxin B1 (AFB1), ergosterol and plate counts measured in rough rice under various storage conditions for up to 15 days

Run	Autoclave	Factors					AFB1 (µg/g)	Ergosterol (µg/g)	Plate count (LogCFU/g)
		a	b	c	d	e			
1	No	12	control	30	95	9	ND	39.043	6.851
2	No	12	10^4	20	75	15	ND	15.766	4.753
3	No	12	10^6	20	95	15	ND	17.113	5.017
4	No	12	10^6	30	85	3	0.066	15.715	5.699
5	No	15	control	20	75	3	ND	12.201	5.199
6	No	15	10^4	25	85	9	ND	16.225	5.672
7	No	18	control	25	95	15	ND	15.766	6.252
8	No	18	10^6	20	95	3	ND	18.133	5.758
9	No	18	10^6	30	75	15	ND	13.324	4.457
10	Yes	12	control	20	95	3	ND	11.932	NG
11	Yes	12	control	30	75	15	ND	13.422	NG
12	Yes	12	10^6	25	75	3	ND	13.085	3.195
13	Yes	15	10^4	25	85	9	ND	15.618	2.954
14	Yes	15	10^6	30	95	15	ND	50	6.426
15	Yes	18	control	20	85	15	ND	12.159	NG
16	Yes	18	control	30	75	3	ND	13.422	NG
17	Yes	18	10^4	30	95	3	0.03	12.133	3.392
18	Yes	18	10^6	20	75	9	ND	13.507	2.479

[2] Factors are denoted by letters; a = storage MC (%), b = mold load (spores/mL), c = storage temperature (°C), d = storage relative humidity (%), e = storage duration (Days)
[3] ND = not detected
[4] NG = no growth

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3
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Table 5: The concentrations of Aflatoxin B1 (AFB1), ergosterol and plate counts measured in brown rice under various storage conditions for up to 15 days

Run	Autoclave	Factors					AFB1 (µg/g)	Ergosterol (µg/g)	Plate count (LogCFU/g)
		a	b	c	d	e			
1	No	12	control	30	95	9	0.114	3.182	6.660
2	No	12	10^4	20	75	15	0.024	3.430	3.491
3	No	12	10^6	20	95	15	0.01	19.464	6.620
4	No	12	10^6	30	85	3	0.018	24.571	4.342
5	No	12	control	20	75	3	0.03	13.428	4.054
6	No	12	10^4	25	85	9	0.007	5.180	5.149
7	No	12	control	25	95	15	0.1	42.516	6.929
8	No	12	10^6	20	95	3	0.036	34.670	3.669
9	No	12	10^6	30	75	15	9.198	92.57	6.349
10	Yes	12	control	20	95	3	ND	2.867	NG
11	Yes	12	control	30	75	15	ND	4.826	NG
12	Yes	12	10^6	25	75	3	ND	2.992	2.904
13	Yes	12	10^4	25	85	9	ND	3.433	3.000
14	Yes	12	10^6	30	95	15	4.908	75.401	6.609
15	Yes	12	control	20	85	15	ND	4.021	NG
16	Yes	12	control	30	75	3	ND	3.418	NG
17	Yes	12	10^4	30	95	3	ND	10.893	4.195
18	Yes	12	10^6	20	75	9	ND	5.353	3.301

[5] Factors are denoted by letters; a = storage MC (%), b = mold load (spores/mL), c = storage temperature (°C), d = storage relative humidity (%), e = storage duration (Days)
[6] ND = not detected
[7] NG = no growth

Table 6: The concentrations of Aflatoxin B1 (AFB1), ergosterol and plate counts measured in milled rice under various storage conditions for up to 15 days

Run	Autoclave	Factors					AFB1 (µg/g)	Ergosterol (µg/g)	Plate count (LogCFU/g)
		a	b	c	d	e			
1	No	12	control	30	95	9	0.240	51.77	6.477
2	No	12	10^4	20	75	15	0.126	0.921	2.303
3	No	12	10^6	20	95	15	0.018	5.334	5.778
4	No	12	10^6	30	85	3	0.03	24.438	3.678
5	No	12	control	20	75	3	0.024	0.921	2.004
6	No	12	10^4	25	85	9	0.024	5.334	5.061
7	No	12	control	25	95	15	0.198	24.438	6.855
8	No	12	10^6	20	95	3	0.03	0.968	3.167
9	No	12	10^6	30	75	15	0.1	24.571	6.591
10	Yes	12	control	20	95	3	0.036	0.924	NG
11	Yes	12	control	30	75	15	0.024	1.030	NG
12	Yes	12	10^6	25	75	3	0.024	0.943	3.222
13	Yes	12	10^4	25	85	9	0.036	0.924	2.885
14	Yes	12	10^6	30	95	15	0.612	48.934	4.797
15	Yes	12	control	20	85	15	0.024	1.03	NG
16	Yes	12	control	30	75	3	0.024	0.861	NG
17	Yes	12	10^4	30	95	3	0.072	22.738	4.204
18	Yes	12	10^6	20	75	9	0.3	0.921	3.740

[8] Factors are denoted by letters; a = storage MC (%), b = mold load (spores/mL), c = storage temperature (°C), d = storage relative humidity(%), e = storage duration (Days)
[9] ND = not detected
[10] NG = no growth

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DEGREE OF ERGOSTEROL AND AFB1 PRODUCTION IN EXTREME CONDITIONS

After identifying the significant factors through screening analyses, the subsequent phase of the study aimed to evaluate the influence of these factors on the production of AFB1 and ergosterol under extreme conditions. The purpose was to examine the effects of these factors at their highest or lowest levels to better understand their impact on the synthesis of AFB1 and ergosterol, which are important indicators of fungal activity and toxin production. During the study, the levels of ergosterol and AFB1 were monitored in brown and rough rice stored at 20°C and 30°C with a constant relative humidity (RH) of 95% throughout the storage periods (day 0, 20, 40 and 60). The initial concentrations of ergosterol in the rough and brown rice samples were 2.72 μg/g and 0.45 μg/g, respectively. These values fell within the range estimated for good-quality cereal grains according to Pietri et al. (2004), who suggested that concentrations should be less than 3 μg/g, with levels higher than 8-15 μg/g considered moldy grains. Our findings were consistent with this assessment (Ng et al., 2008).

Over the storage period, ergosterol production gradually increased in both rice fractions when stored at 20°C, peaking at day 60, as depicted in Figure 11. Initially, low levels of ergosterol in the first few days indicated that the fungi were adapting to the new storage environment, and thus no significant increase in fungal biomass occurred. However, with an extended duration of storage up to day 60, ergosterol levels increased, possibly to maintain the integrity of the fungal membrane which is essential during the growth phase (Hu et al., 2017). It is worth noting, however, that the concentration of ergosterol observed in brown rice during this storage period and condition was significantly higher (154 μg/g) when compared to rough rice (48.2 μg/g). This disparity can be attributed to the nutritional components present in brown rice, which, as previously stated, provided a favorable food source for fungal growth, leading to higher ergosterol levels compared

to rough rice. This finding aligns with the discovery made by Taniwaki et al. (2006), who found that certain fungal species produced higher levels of ergosterol on Potato Dextrose Agar (PDA) than on Czapek Yeast Extract Agar (CYA). From this, we can infer that the composition of the substrate can influence ergosterol production.

In contrast, when the rice samples were stored at 30°C, ergosterol levels increased and then declined towards the end of the storage period (Fig 11). At this temperature, the highest ergosterol levels in rough (56.1 µg/g) and brown (38.7 µg/g) rice were observed after 20 and 40 days, respectively. This pattern is consistent with the observations made by Gourama et al. (1995), who reported a similar trend with ergosterol levels increasing to a maximum and then declining towards the end of the incubation period. Generally, fungal growth is known to have a lag phase followed by an exponential growth phase (Garcia et al., 2009). The stationary phase appears only when the conditions are suboptimal, this is when the rate of cell division balancing out the rate of cell death over time (Garcia et al., 2009). Presumably, the decline of ergosterol towards the end could be attributed to a decrease in fungal biomass, which results from depletion of resources and nutrients. Bearing in mind that during this phase, death exceeds growth, ergosterol begins to degrade with age (Kadakal et al., 2019). This assumption aligns with the findings of another study that observed lower ergosterol levels following the autolysis of mycelium and concluded that ergosterol degraded in the dead hyphae (Ekblad et al., 1998).

There was a consistent pattern in AFB1 production in the rice samples when stored at 30°C. The highest AFB1 levels detected in both rough and brown rice were observed after 20 days of incubation and then declined towards the end of the storage period, as shown in Figure 12. These findings are consistent with previous studies that also reported similar trends in AFB1 production in rice samples (Gqaleni et al., 1997; Lahouar et al., 2016; Castro et al., 2002; Liu et al., 2006). In

contrast, when the rice samples were stored at 20°C, the levels of AFB1 were much lower. In brown rice, AFB1 was detected on day 20 (0.013 µg/g), slightly increased on day 40 (0.081 µg/g), and then decreased on day 60 (0.025 µg/g). On the other hand, AFB1 in rough rice was only detected on day 20 (0.393 µg/g) and day 60 (0.108 µg/g). While the exact explanation for the degradation of AFB1 during storage remains unclear, some researchers have proposed a direct link between the mold and the degradation process (Lahouar et al., 2016). Additional studies have shown that certain species of *Aspergillus* and *Rhizopus* can break down aflatoxin into a less toxic metabolite called aflatoxicol (Wu et al., 2009). This discovery extends beyond fungal communities and includes various microorganisms, such as bacteria found in soil, demonstrating the diverse range of organisms capable of degrading aflatoxins (Wu et al., 2009).

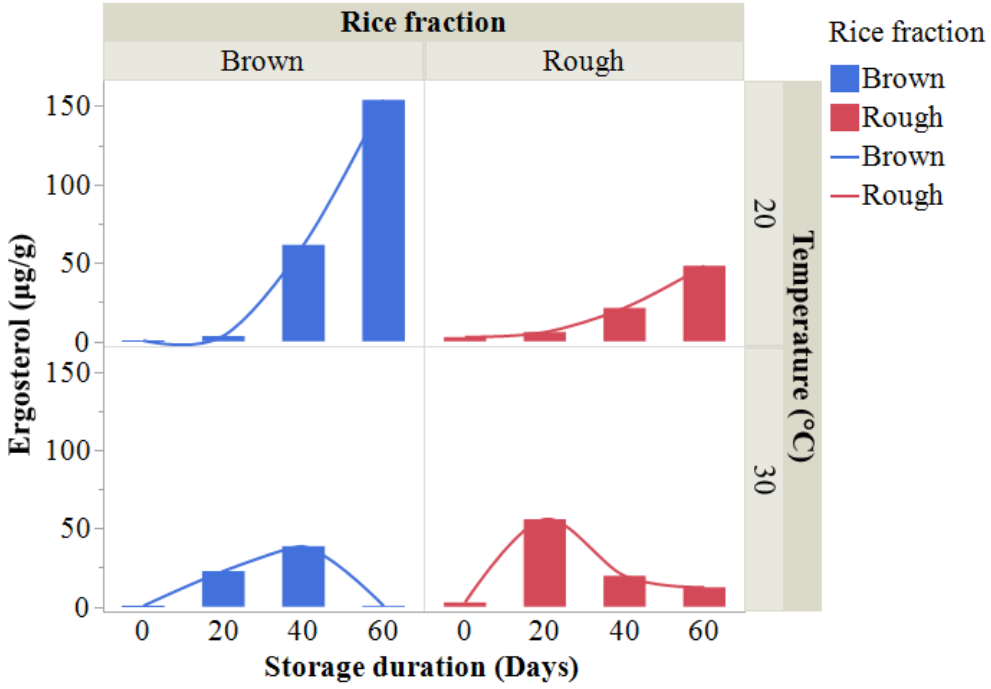

Figure 11.Changes in ergosterol production in rough and brown rice over 60 days storage at 20°C and 30°C with constant RH of 95%.

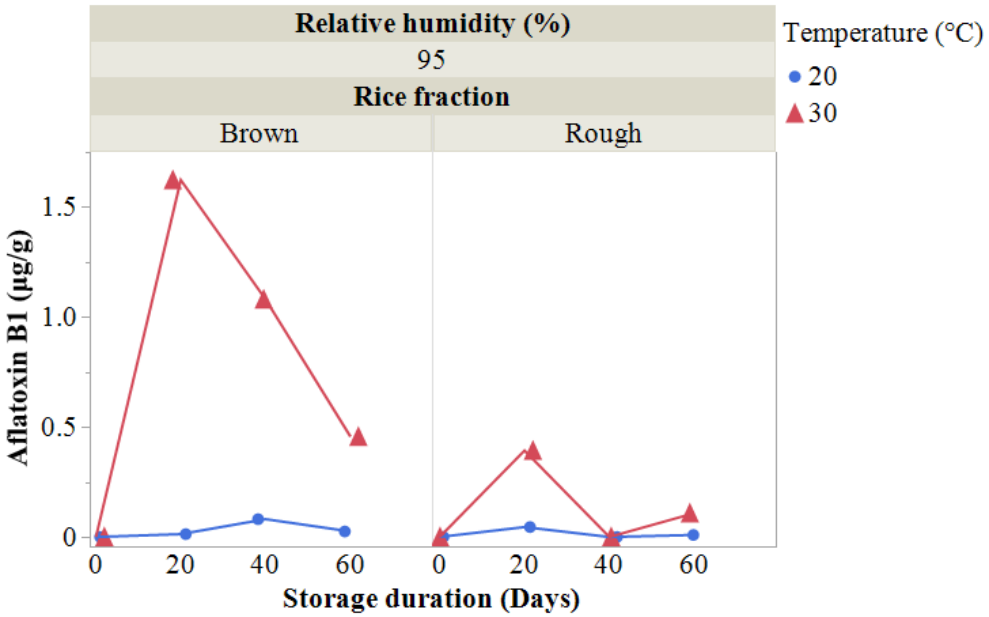

Figure 12. Changes in aflatoxin production in rough and brown rice over 60-day storage at 20°C and 30°C with constant RH of 95%.

CONCLUSIONS

Temperature and relative humidity were found to be the most important factors influencing the growth of *A.flavus* and AFB1 production in different rice fractions. Fungal growth and AFB1 production occurred in milled rice over a wider range of temperature and relative humidity compared to rough rice. The maximum fungal growth was observed at 25°C and a relative humidity of 95% in milled rice. However, the combination of a lower relative humidity of 75% and a higher temperature of 30°C did not inhibit fungal growth in brown and milled rice. AFB1 production was dependent on rice fraction with decreasing levels observed as the storage duration increased up to 60 days. Brown rice is more susceptible to fungal spoilage and aflatoxin contamination than milled or rough rice. Importantly, the conditions that promote fungal growth may not always be ideal for AFB1 production. Therefore, these findings provide valuable insights that can assist in implementing preharvest interventions to minimize fungal spoilage and AFB1 contamination in rice and rice products.

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PROJECT CONCLUSIONS

This study mainly focused on securing microbial safety of rice. The first part of the study explored the use of microwaves as an alternative drying technology to convective drying methods, which could significantly reduce the risk of aflatoxin contamination in rice by inactivating toxigenic fungi responsible for aflatoxin contamination. The study employed an industrial microwave system operating at 915 MHz to dry long grain rice (variety RT 7321) using high powers and short-duration heating. The impact of microwave power levels (16, 18, and 20 kW) and heating durations (1, 2, and 3 minutes) on the reduction of microbial load in rice with a bed thickness of 20 mm was investigated. Results showed that at 20 kW and a heating duration of 3 minutes (corresponding to a specific energy of 1200 kJ/kg), there was a significant reduction in fungi (5.01 CFU/g) and aerobic bacteria (1.2 CFU/g).

The second part of the study addressed how storage conditions affected the growth of toxigenic fungi, *Aspergillus flavus*, and the production of AFB1 in rough, brown, and milled rice, specifically because contamination of the grains with aflatoxins is largely due to improper post-harvest storage. Temperature and relative humidity were found to have a substantial impact on the growth of *A. flavus* and AFB1 production. Optimal conditions for *A. flavus* growth were observed at 30°C and 95% relative humidity. The production of AFB1 depended on the type of rice and storage conditions, with brown rice being more susceptible to fungal spoilage and aflatoxin contamination compared to milled or rough rice.

These findings are of great relevance because they address critical issues faced by farmers to help mitigate aflatoxin contamination in rice. This can significantly improve rice safety and contribute to economic development by reducing the economic losses associated with aflatoxin contamination. In addition, identifying safe storage conditions will have a profound impact on

agricultural practices by improving post-harvest storage and handling methods to minimize the risk for subsequent aflatoxin contamination. Moreover, utilizing microwaves as a drying technology can contribute to sustainable energy sources, making the process more efficient and cost-effective. Overall, the control of aflatoxins in crops is a critical area of research that requires multidisciplinary efforts to develop sustainable and effective solutions to mitigate this problem.