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Comparative Occurrence of Resident Fungi on Gamma Irradiated and Steam Sterilized Sorghum Grains (*Sorghum bicolor* L.) for Spawn Production in Ghana

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NKK and GTO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors VA and MO managed the analyses of the study. Authors MWK and AAG managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Sorghum is one of the important cereals consumed by humans, animals and also used for the production of mushroom spawns in Ghana.
Aim: Identification of fungi present on sorghum grains before and after pretreatment (steam and gamma radiation) principally for mushroom cultivation.

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Methodology: The total number of mycoflora (Log_{10} CFU g^{-1}) of sorghum grains and their relative frequency (percentage occurrence) associated with the raw grains and the mycoflora present after subjecting the sorghum grains to gamma radiation doses of 0, 5, 10, 15, 20, 25 and 32 kGy at a dose rate of 1.7 kGy/h from a Cobalt-60 source (SLL-515, Hungary) and moist heat at a temperature of 100- 120ºC for 2- 2.5 hours was evaluated. Mycological analysis was done by direct plating method on Cooke's and Dichloran Rose Bengal Chloramphenicol (DRBC) media.
Results: Nine fungal species belonging to six genera were associated with the sorghum grains. Among these fungi were *Cladosporium macrocarpum, Trichoderma harzianum, Fusarium oxysporum, Rhodotorula* spp., *Penicillium* spp., *Aspergillus niger, Aspergillus fumigatus, Aspergillus ochraceous* and *Aspergillus flavus.* Comparatively higher fungal counts of 3.27 and 3.82 Log_{10} CFU g^{-1} were recorded for non-pretreated while lower counts of 0.5 Log_{10} CFU g^{-1} were recorded for pretreated sorghum grains. Gamma radiation and moist heat significantly ($P<0.05$) reduced total fungal populations by an average of 2.4 and 2.1 log cycles, respectively. *Rhodotorula* sp. (11.5%), *Penicillium* sp. (34.6%), *Aspergillus fumigatus* (29.9%) persisted on the moist heat sterilized while only *Rhodotorula* sp. (100%) persisted on gamma irradiated grains.
Conclusion: These data indicate possible health hazards for humans and animals upon consumption of such contaminated food grain by toxigenic moulds and also reveal the sensitivity of fungal species to gamma radiation and moist heat as a selective substrate for oyster mushroom spawn preparation.

Keywords: Sorghum; gamma radiation; steam; D10 value; fungi; mushroom.

1. INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal after rice, wheat, maize and barley [1]. It is the staple food grain for over 750 million people who live in the semi-arid tropics of Africa, Asia, and Latin America [2,3]. The sorghum crop is still a principal source of energy, protein, vitamins and minerals for millions of poor people in these regions. Besides its traditional use as food crop, sorghum has other alternative uses such as livestock and poultry feed, potable alcohol, starch, ethanol production, numerous industrial purposes [4]. Significantly among the list is its usage for mushroom spawn production.

Numerous fungi associated with sorghum grains are implicated as macro / micro organisms responsible for competition with the mycelium of the cultivated mushroom. The source of contamination is largely dependent on such factors as place of origin, physiological maturity, storage quality, grain density are of serious concern due to their fungi toxigenic potential. Again, some major effects of fungal deterioration of grains include decreased germination, discoloration, development of visible mold growth, musty or sour odors, dry matter loss and nutritional heating, caking, and the potential for production of mycotoxins in the grain. According to [5], the toxigenic moulds commonly isolated from foods or grains are *Aspergillus*, *Penicillium* and *Fusarium*. In storage conditions, *Aspergillus* and *Penicillium* are predominant and the *Fusarium* spp. is an important plant pathogen. Aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) are mycotoxins produced by *Aspergillus flavus (*AFB_1 and AFB_2 producer) and *A. parasiticus* (AFB_1, AFB_2, AFG_1 and AFG_2 producer). These species are commonly recognized in grains as maize or peanuts. Aflatoxin B1 is most toxic of the group followed in decreasing toxicity by AFG_1, AFB_2 and AFG_2. Aflatoxins are recognized in some species as responsible for toxic signs and lesions, reduced growth, immune suppression and liver cancer [6,7]. The International Agency for Research on Cancer has classified AFB_1 as a probable human carcinogen [8].

In Ghana, *Pleurotus ostreatus* (Jacq. Ex. Fr) Kummer, strain EM-1, is the most cultivated mushroom [9]. The spawns of this species of mushroom has been prepared using moist heat sterilized sorghum grains. The spawn which is often the innoculum is a network of pure culture of fungal vegetative tissues interweaves a medium such as cereal grain [10,11,12]. Published works reveals that different media has been used for spawn production such as wheat [13,14,15], rye [14], sorghum [14,15], rice [16], millet [16,13,15] and white maize [15]. Essentially, these materials serve as a propagative media for the cultivation of mushroom. Failure to achieve a satisfactory harvest may often be traced to unsatisfactory spawn used [14]. Mushroom cultivation serves as the most efficient and economically viable

biotechnology for the conversion of lignocellulose waste materials into high quality protein food per unit area [17] and this will naturally open up new job opportunities, especially in rural areas, urban and peri-urban areas in this golden age of enterpreneurship.

Gamma irradiation as a physical treatment effectively eliminates spoilage and pathogenic microorganisms in foods [18,19,20,21] and has been utilized for the reduction and elimination of pathogens in foods [22,23]. However in order to utilize irradiation as a food processing technology, it is imperative to study the radiation sensitivity of contaminating microorganisms since this provides a basis for accurate estimation of inactivation doses [24,25]. Sensitivity to irradiation varies among microbial and fungal species and is affected by the components of foods and temperature during irradiation and subsequent storage [26,27]. The D_{10}-value (decimal reduction dose) is the radiation dose required to inactivate 90% of a viable bacterial population or reduce the population by a factor of 10 [28]. There is a comparatively great range of D_{10}-values and therefore differences in resistance to gamma radiation by various microorganisms of public health significance. Published data [29,30] on D_{10} values for *Aspergillus flavus* was 0.43 and 0.5 kGy in buffered saline solution and in smoked herrings, respectively.

Studies on the relative radiation-resistant fungal species by Abouzeid et al. [29] illustrated that *Aspergillus* and *Penicillium* species are relatively sensitive to ionizing radiation with a D_{10} values between 0.25 and 0.65 kGy whereas other species in the genus *Fusarium* are more resistant requiring high but safe D_{10} v alues of 0.65 to 1.5 kGy. Estimation of D_{10}-values may be incorporated into risk assessments for designing processes for reduction of microbial populations in food [31].

This paper seeks to assess the mycofloral population, species diversity and compare the effect of gamma irradiation and moist heat sterilization on the mycofloral population of sorghum grains for spawn preparation.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Sorghum samples (approximately 1000 g) were collected from Madina market, Accra, Ghana in 2013. Samples were brought to the laboratory in sterile plastic bags and kept at 4ºC. All the samples were subjected to mycological analysis.

2.2 Moist Heat Sterilization

Grains were steeped in water overnight for about 12 hours. About 265 g of grains were packed into bottles and then transferred into transparent heat resistant polypropylene bags (24 cm x 38 cm) and then plugged with cotton wool and covered with plain sheets. The sheets were held in place with rubber bands. The grains were sterilized in an autoclave (Priorclave, Model PS/LAC/EH150, England) at 121ºC for 1h.

2.3 Irradiation

Sorghum grains were soaked overnight and packaged as described above and then irradiated at doses 0, 5, 10, 15, 20, 25 and 32 kGy at a dose rate of 1.7 kGy per hour in air from a cobalt 60 source (SLL 515, Hungary). Doses were confirmed using the ethanol-chlorobenzene (ECB) dosimetry system at the Radiation Technology Centre of the Ghana Atomic Energy Commission, Accra, Ghana.

2.4 Determination of pH

According to AOAC [32].

2.5 Determination of Moisture Content

According to AOAC [32]

2.6 Enumeration of Mycoflora

The dilution plate technique was used in estimating fungal populations. About 10 g fresh weight of sample was placed in 250 ml Erlenmeyer flask containing 100 ml sterile distilled water. The mixture was shaken at 140 rev. /min in a Gallenkamp Orbital Shaker for 30 min. Aliquot (1 ml) of the suspension was placed in sterile universal bottles (MaCartney tubes) containing 9 ml of 0.1% peptone, and was serially diluted up to $1:10^{-3}$. The fungal population was enumerated on modified Cooke's medium [33] and Dichloran Rose Bengal Chloramphenicol (DRBC) agar incubated at 30-32ºC for 5 to 7 days for species diversity.

2.7 Characterization and Identification of Fungal Isolates

Fungal isolates were examined under stereo-binocular microscope (Leica 261, Germany)

using the needle mounts technique. Their identification was performed according to macro and micro morphological characteristics. All the isolates were identified up to the species using keys and manuals [34,35,36]. The percentage (%) occurrence of fungi was calculated by the formular according to Sreenivasa et al. [37].

Percentage (%) occurence of fungal species=

$$\frac{\text{Number of fungal species isolated}}{\text{Total number of fungi isolated}} \times 100$$

2.8 D_{10} Values Determination

The D_{10} value is the reciprocal of the slope of the exponential part of a survival curve. This value may also be obtained from equation (1). The data was subjected to regression analysis. The surviving fractions, $\log_{10}$ (N/N_0) of microorganisms, was calculated and used as relative changes of their actual viable cell counts. The D_{10} values were calculated by plotting $\log_{10}$ (N/N_0) against dose (D) according to the equation

$$D_{10} = \frac{\text{Radiation Dose (D)}}{\log_{10}(No - N)}$$

Where No is the initial viable count; N is the viable count after irradiation with dose D; D is the radiation dose [38,27]. The linear correlation coefficient (r^2) and the regression equations were also calculated.

2.9 Statistical Analysis

The values obtained for total fungal counts were transformed to logarithm conversions and subjected to analysis of variance (ANOVA) using SPSS (Chicago, IL) version 9 for windows.

3. RESULTS AND DISCUSSION

Results of the influence of gamma radiation and moist heat sterilization (steam) on the relative abundance and total microbial population on the surface of the sorghum grains indicated some significant ($P<0.05$) difference. Both methods of pretreatments were effective in reducing the microbial load. The non-pretreated (control) sample harbored comparatively higher fungal counts of 3.27 and 3.82 $\log_{10}$ CFU/g enumerated from the Cooke's and DRBC growth media, respectively, (Fig. 1). Gamma radiation dose of 5kGy was able to reduce the mycofloral population by 1.2 and 1.6 log cycles while 10kGy recorded 1.7 and 1.86 log cycles respectively for the two growth media. Dose 15 kGy reduced the total fungal populations by 1.7 and 3.1 log cycles for the two growth media, 20 kGy by 2.4 and 3.2 log cycles, 25 kGy by 2.4 and 3.3 log cycles, 32 kGy 2.8 and 3.3 log cycles. The effect of doses 5 kGy, 10 kGy and 15 kGy on the total fungal population enumerated on Cooke's medium showed no significant ($P>0.05$) differences. Likewise, doses 20 kGy, 25 kGy and 32 kGy were also not significantly ($P>0.05$) different. Essentially doses 5, 10, 15, 20, 25, 32 kGy differed ($P<0.05$) significantly from the non-pretreated (control) sample. This observation could be attributed to the production of free radicals by ionizing energy of the gamma radiations which cause injuries to the cells and ultimately death of microorganisms [39,21].

Steam sterilization was also effective in reducing the total fungal population by 1.6 and 2.6 log cycles, respectively, for the two growth media. The effectiveness of steam sterilization in reducing the total fungal population was comparable to doses 10 kGy and 15 kGy. Doses beyond 15 kGy further reduced the total fungal populations to range 0.9- 0.5 $\log_{10}$ CFU/g on Cooke's medium. However on DRBC, steam sterilization reduced total fungal population to 1.23 $\log_{10}$ CFU/g which corresponds to an intermediary of 10 and 15 kGy. Statistically, there were no significant ($P>0.05$) differences recorded between steam sterilization and gamma radiation doses of 10, 15, 20, 25 and 32 kGy (Fig. 1).

Results obtained corroborate results reported by Rico et al. [40] who observed a 1-to-2 log cycle reduction in initial microbial count of 10^6 CFU/g with steam, while gamma irradiation at 10 kGy resulted in a 5-log cycle reduction in same initial microbial count as they investigated the comparative effect of steaming and irradiation on the physicochemical and microbiological properties of dried red pepper (*Capsicum annum* L.). In a similar work, Al-Bachir and Al-Dawi [41] reported a 1-to-2 log cycle reduction in total aerobic plate count with steam while a 4-log cycle reduction was recorded with 10 kGy dose of gamma radiation as they compared the effect of gamma irradiation and heating on the microbiological properties of licorice (*Glycyrrhiza glabra* L.) root powders.

Radiation sensitivity of fungi isolated from sorghum grains cultured on Cooke's and DRBC growth media were 7.9±kGy and 6.4±kGy respectively (Fig. 2 and Table 1). Radiation sensitivity (the killing effect of radiation) in

microorganisms is generally expressed by the decimal reduction dose or D_{10} value [38]. From the calculated D_{10} values in Table 1, it is obvious that the fungal spores were quite radiosensitive in sorghum grains as values obtained are in agreement with published works of Frazier and Westhoff [42] reported D_{10}-values of range 4- 11 kGy for yeasts and 1.3- 11 kGy for moulds.

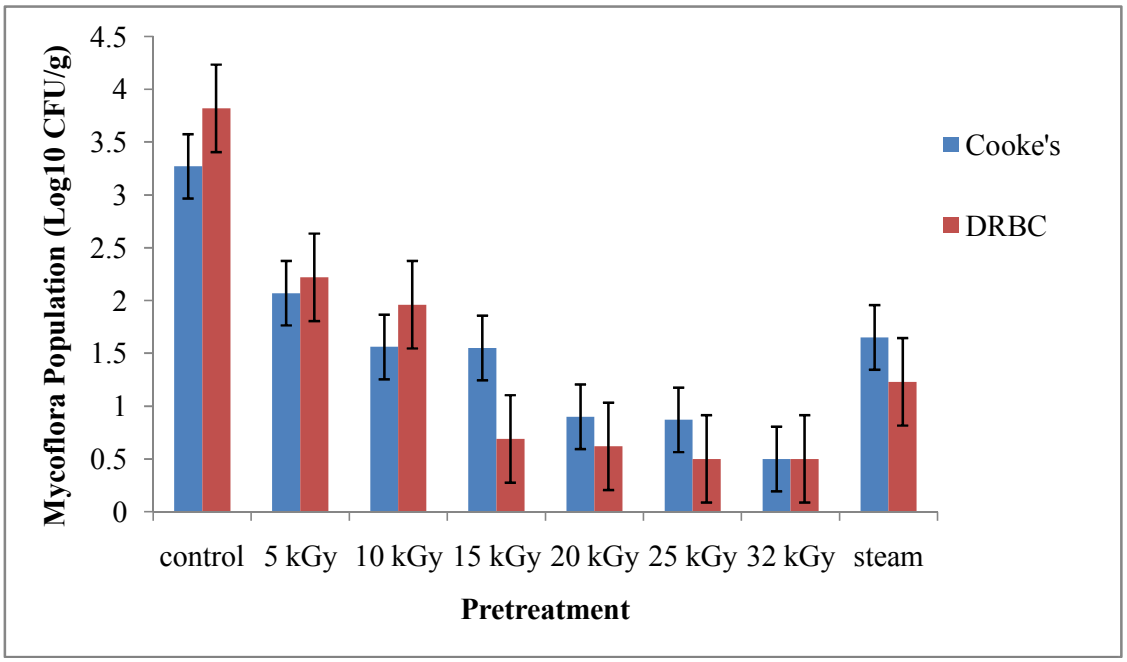

Fig. 1. Mycofloral population of Sorghum grains enumerated from two growth media

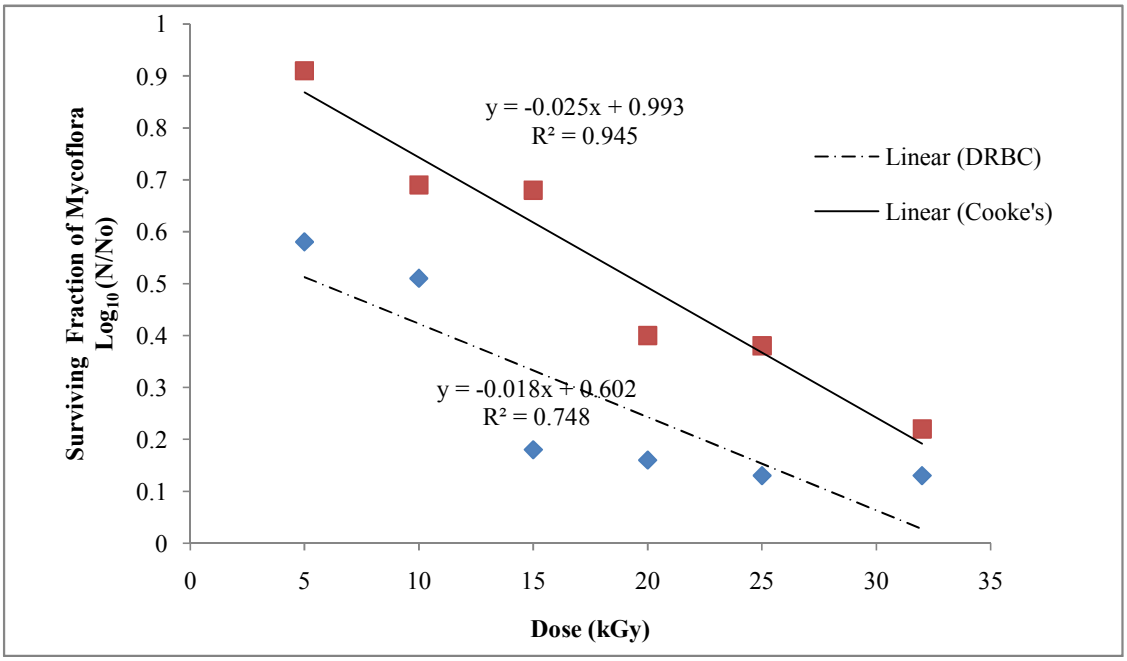

Fig. 2. Radiation sensitivity curves for mycoflora of sorghum grains cultured on 2 growth media

Table 1. Mean D_{10} values of fungi associated with sorghum grains isolated from the two (2) growth media

Substrate	Regression equation	r^2	D_{10} value (kGy)
(a) Cooke's	-0.025x + 0.993	0.945	7.9±1.6
(b) DRBC	-0.018x + 0.602	0.748	6.4±1.3

D_{10} values are means of 6 replicates ± S.E

3.1 Phenology of Mycofloral Population

Various fungi isolated from non-pretreated (control) sorghum grains on Cooke's medium included *Cladosporium macrocarpum* (14.5%), *Trichoderma harzianum* (21.25%), *Fusarium oxysporum* (2.75%), *Rhodotorula* spp. (1.25%), *Penicillium* spp. (7.5%), *Aspergillus niger* (13.75%), *Aspergillus fumigatus* (26.25%), *Aspergillus ochraceous* (2.75%) and *Aspergillus flavus* (10.0%) (Fig. 3). Fungi enumerated from DRBC also included *Fusarium oxysporum* (8.33%), *Penicillium* spp. (28.33%), *Aspergillus niger* (34.14%), *Aspergillus fumigatus* (16.67%), *Aspergillus flavus* (8.33%) and *Aspergillus ochraceous* (4.17%) (Fig. 4).

The dynamics of a fungal community may be attributed generally to abiotic variables and nature of substrate [43]. Pretreatment of sorghum grains resulted in the disappearance and appearance of certain fungal species which was recorded as the percentage occurrence of the fungal species relative to the total population / number of species recorded. Antagonism between fungi according to Obodai and Odamtten [44] may be in the form of competition for nutrients, chemical antibiosis and lysis of mycelia. Antibiosis is the inhibition of one generation by the metabolic product of another. Although it is usually an inhibition of growth and sporulation, it may be lethal. Metabolites produced by protagonist fungi penetrate the cell wall of the antagonist and inhibit activity by chemical toxicity [45].

The vulnerability of microorganisms and their spores to gamma radiation has been well recognized by researchers [46,25,27,21]. The ionizing radiation produces chemical changes on substrate that inactivate microorganisms. Many applications are realized also to reduce the microorganism number and consequently eliminate the risks of a poisoning disease. The energy of ionizing radiation affects directly the microbial DNA molecules, causing the damage on fungal or bacterial cells. The ability of an organism to withstand a physical stress (gamma radiation and/or steam) depends on how quickly it is able to repair its damaged DNA as a result of denaturing [47].

Generally on both growth media, fungal populations and species number decreased as

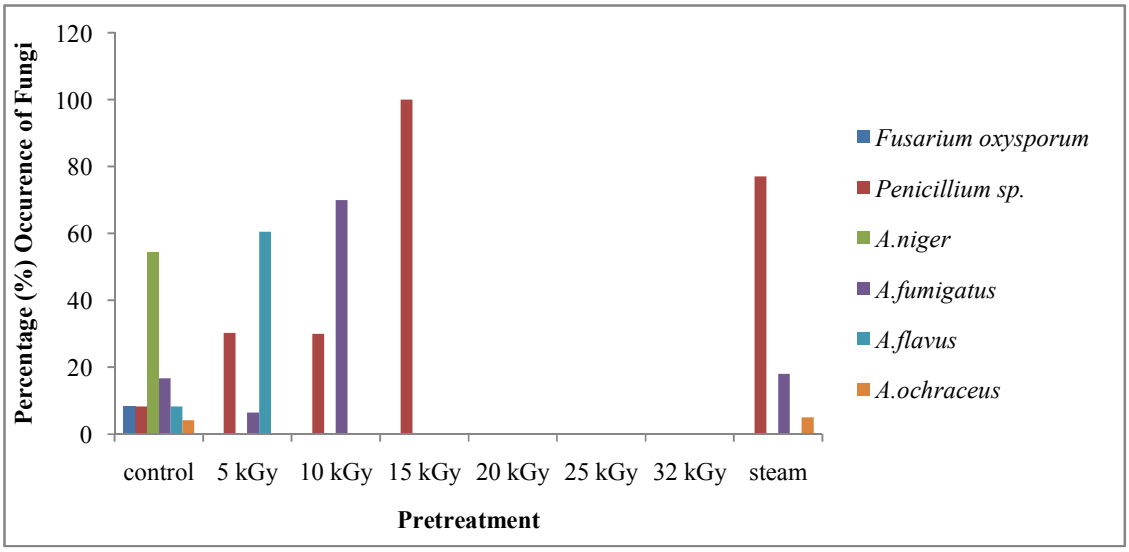

Fig. 3. Mycoflora isolated on DRBC medium after pretreatment of sorghum grains

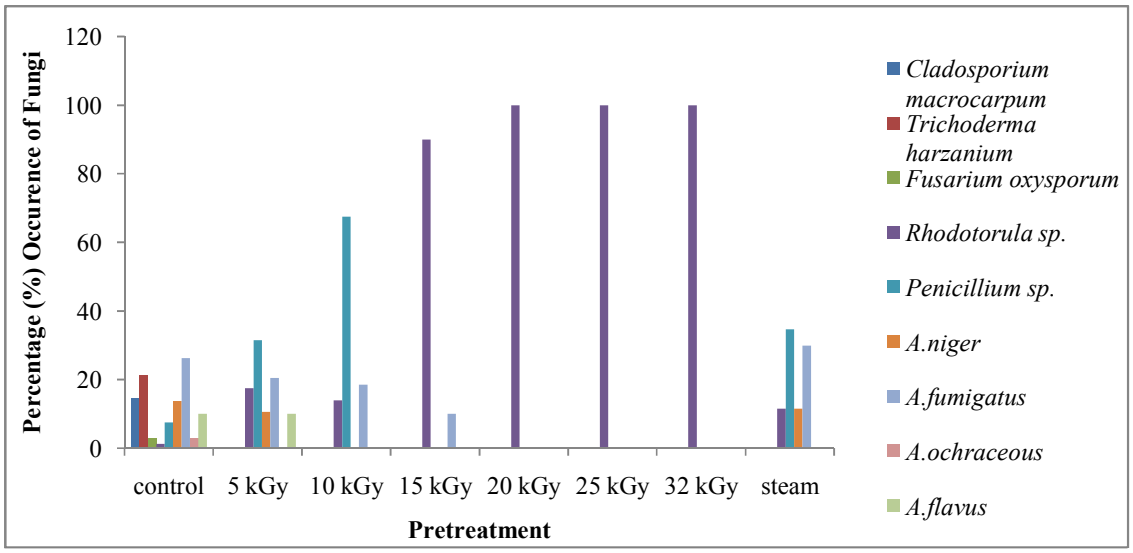

Fig. 4. Mycoflora isolated on cooke's medium after pretreatment of sorghum grains

gamma radiation doses increased. At gamma radiation dose 10 kGy, *A. fumigatus* (30%) and *Penicillium* spp. (70%) persisted. Also only *Rhodoturula* spp. (100%) persisted at 15kGy. Nonetheless, doses beyond 15kGy recorded no microorganism growth on DRBC.

Similarly on Cooke's medium, *Rhodotorula* spp. (14.0%), *Penicillium* spp. (67.5%), and *Aspergillus fumigatus* (18.5%) persisted at 10 kGy. At 15 kGy, *A. fumigatus* (10.0%) and Rhodotorula spp. (90.0%). However, beyond 15 kGy no fungi survived except Rhodotorula spp. (100.0%). Steam sterilized sorghum grains harbored *Penicillium* spp. (77.0%), *Aspergillus fumigatus* (18.0%) and *Aspergillus ochraceous* (5.0%) enumerated from DRBC. While *Rhodotorula* spp. (11.54%), *Penicillium* spp. (34.62%), *Aspergillus niger* (11.54%) and *Aspergillus fumigatus* (29.9%) were enumerated from Cooke's medium.

The variation in resistance of adverse conditions such as gamma radiation, steam, drought etc. in filamentous fungus strains can be explained by multiple factors. The cell walls of some fungi contain appreciable fractions of lipids (up to 20%) as in the case of some *Aspergillus* species. Some investigators postulated that filamentous fungi produce numerous metabolites, such as alcohols, acids, enzymes, pigments, polysaccharides, and steroids, as well as some complex compounds, such as ergotinine, and antibiotics, including penicillin, notatin, flavicin, and fumigacin. In addition, intracellular fungal components (sulfhydric compounds, pigments, amino acids, proteins and fatty acids) have been reported to be responsible for radioresistance of fungi [48]. Aquino et al. [30] demonstrated a higher resistance of the *Aspergillus flavus* to gamma radiation, which showed no growth after exposure to 10 kGy.

The genus *Aspergillus* was the most dominant fungi among 10 fungi reported in this study. It was reported as a natural contaminant in cereals and also in many other agricultural commodities in previous studies by Hocking, [49] and Thakur et al. [50]. Mycological studies conducted on sorghum by Sreenivasa et al. [36] revealed that sorghum was contaminated by nine species of *Aspergillus* (Table 2). The predominant *Aspergillus* species isolated were *A. flavus* (72.7%) and *A. niger* (59.1%) with the relative percentage of 51.1 and 33.3%, respectively. The three *Aspergillus* species such as *A. ochraceus, A. versicolor* and *A. candidus* were recorded with a similar frequency of 20.5%. A low frequency of *A. sydowii* (2.3%) was recorded in Table 2. Surveys conducted worldwide also revealed that, *A. flavus* and *A. niger* were known to frequently contaminate peanuts and were able to produce mycotoxins such as aflatoxins [51,52,53,54]. *A. flavus* contamination and aflatoxin production in sorghum is a serious problem in most of sorghum producing countries where the crop is grown under rain fed conditions [54]. Fungi isolated in this work were common to previous mycological works by some researchers [55,56,57] on sorghum grains.

3.2 Moisture Content (%) and pH

In the present study, moisture content ranged 18.21±0.78 - 18.85±0.65% for sorghum grains irradiated at doses 10 kGy and 32 kGy and also for control, respectively, (Table 2) which apparently supported growth of a wide range of fungal diversity as well as load of > 10^3 CFU/g. Higher moisture content makes a substrate favorable for fungal invasion [58]. This is in direct agreement with the findings of Quezada et al. [59] who reported a gradual increase in fungal load and diversity with an increase in moisture content of stored maize sample. Moisture content along with substrate type and nutrient availability and presence of secondary metabolites also affect the extent of fungal contamination [60,61]. Essentially, moisture content which is too high (> 65%) could cause oxygen depletion and losses of nutrients through leaching [44]. On the other hand, low moisture content below a critical level (< 30%), would decrease activities of microorganisms by restricting the motility and make them dormant [62]. Under drier conditions, the ammonium and ammonia present generate a higher vapor pressure; thus conditions are more favorable for nitrogen loss.

The hydrogen environment of fungi is difficult to study because fungi change the pH as they grow. Some species increase and others decrease pH of their medium. pH of the medium is important because it influences mineral availability, enzyme activity and membrane function. Generally speaking, fungi can tolerate a wide range of pH, though most media used to culture fungi are acidic. During present investigation, samples with a low pH range (5.61±0.05 - 6.36±0.04) were found to harbor a good number of fungi. Reports of [12,44] indicate that low pH (acidic range pH 4– 6) favors good fungal growth and recolonization of fungi [63]. Generally, there were significant differences ($P<0.05$) between the treatments. *Aspergillus* was recorded as the most dominant genus in samples of all pH ranges; this can be attributed to the ability of the *Aspergilli* to grow in a wide range of pH. Wheeler et al. [64] reported that *Aspergillus* species are more tolerant to alkaline pH, while *Penicillium* are more tolerant to acidic pH. This is in accordance with our findings where *A. niger* and *Penicillium* sp. were recorded as the most dominant fungal species in the pH ranges of 3.50 to 7.0. Some scientists [65,12] stated that optimum pH ranges are mainly related to different species, strains, enzymatic systems, important vitamin entry in the cell, mineral capture, and surface metabolic reactions. High pH tends to suppress the growth as well as antagonize certain fungi in compost thus reducing competition for the mushroom [66].

Table. 2. Effect of pretreatment on physical and chemical properties of *Sorghum bicolor*

Treatment	Moisture content (%)	pH
Control	18.85±0.65	6.36±0.08
5 kGy	18.53±0.66	5.67±0.05
10 kGy	18.21±0.38	5.62±0.05
15 kGy	18.27±0.33	5.92±0.05
20 kGy	18.56±0.45	5.65±0.05
25 kGy	18.84±0.64	5.85±0.05
32 kGy	18.85±0.64	5.61±0.05
Steam	18.69±0.62	5.77±0.05

4. CONCLUSION

The use of gamma irradiation treatment is a vital tool for the control of fungal microorganisms in foods and seeds. These products are often consumed raw or in their natural state. Data obtained reveal the type of fungi and an estimate of microbial loads on the sorghum grains as well as the level of reduction obtained when pretreated with steam and gamma radiation. Gamma irradiation proved to be an effective method for the control of microbes and so could be used as an alternative method of sterilization for sorghum spawn preparation. Despite the existence of these sterilization technologies, it is necessary to have a monitoring Program of Good Manufacturing Produce (GMP) and Hygienic practices to avoid fungal contamination during manufacturing process, storage and exposure of products on the market.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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