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Microbial and Fungal Contamination of Staple Foods in Port Harcourt, Nigeria: Special Attention to High Aflatoxin Risk

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HIGHLIGHTS

- Groundnut samples had very high aflatoxin levels in most of the markets in Port Harcourt, Nigeria.
- *Aspergillus flavus* and *A. niger* were isolated from groundnut and maize samples.
- Groundnut and cowpea samples had high bacterial counts in most markets.

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Acronyms and abbreviations
CFU=Colony Forming Unit
MC=Moisture Content

ABSTRACT

Background: Microbial and fungal contamination of agricultural produce has been a health challenge over the years. The present study surveyed microbial and aflatoxin contamination in groundnut, maize, and cowpea collected from Port Harcourt, Nigeria.

Methods: Ninety samples of maize, groundnut, and cowpea were purchased from six major markets in Port Harcourt, Nigeria. The samples were first examined for insect pest infestation, then Moisture Content (MC), microbial, and aflatoxin contamination. Characterization of bacterial isolates was determined based on their morphological and cultural characteristics. Statistical analyses were performed using SPSS 20.0

Results: Data showed that 50% of groundnut samples and 33.33% of maize samples had total aflatoxins levels above World Health Organization (WHO) acceptable limits of 0.5-15 µg/kg. MC for groundnut, maize, and cowpea samples significantly ranged from 2.48-5.55%, 9.00-11.25%, and 9.50-12.48%, respectively. The mean bacterial count for groundnut, maize, and cowpea samples ranged from 0.7×10^8-1.7×10^8 Colony Forming Unit (CFU)/g, 0.3×10^8-1.7×10^8 CFU/g, and 0.7×10^8-1.9×10^8 CFU/g, respectively. Bacterial isolates, including *Pseudomonas* sp., *Streptococcus* sp., and *Clostridium* sp. were isolated from groundnut while *Bacillus* sp., *Staphylococcus* sp., *Proteus* sp., and *Escherichia coli* were isolated from maize and cowpea. Fungal isolates, including *Aspergillus flavus* and *A. niger* were isolated from groundnut and maize.

Conclusion: This study revealed the health risk exposure of consumers of the assayed staples in Port Harcourt of Nigeria, especially groundnut which had very high aflatoxin levels in most of the markets.

Introduction

Food contamination refers to the diverse ways in which the integrity of food has been depraved, and this can be biologically, chemically, and physically (Abdolshahi and Shokrollahi Yancheshmeh, 2020). This includes the

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presence of microorganisms or derived toxic substances such as mycotoxin in food that makes them unsafe for human and animal consumption. Major food contaminants include but are not limited to mycotoxins and other microbial toxins, toxic elements, radioactive isotopes, nitroso compounds, polycyclic aromatic hydrocarbon, halogen containing organic compounds, pesticides residues, veterinary drug residues, etc. (EFSA Panel on Biological Hazards, 2011).

Aflatoxins are forms of mycotoxins which occur naturally and are produced by *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* (El Tawila et al., 2020). They are highly toxic and carcinogenic compounds that have deleterious effect on the health of humans and animals (Benkerroum, 2020). Aflatoxin contamination emanates through diverse routes in the food system from the field to the home, and to the marketplace (Pickova et al., 2021). Prevalence data from Africa suggests that aflatoxin contamination in maize, groundnuts, and sorghum occurs above safe levels in most African countries (Meijer et al., 2021; Nji et al., 2022).

Aflatoxin contamination of agricultural produce has been a challenge over the years especially because of diverse risks associated with the consumption of contaminated agricultural produce, not only to humans, but also to animals (Meneely et al., 2022). Aflatoxins are in the category of Group-1 carcinogens according to International Agency for Research on Cancer (IARC) ratings of carcinogens (IARC, 2012). This implies that it is an established human carcinogen. Studies have revealed the alarming rate of child-aflatoxin exposure in many African countries especially from infancy (Akbari et al., 2017; Wild et al., 2016). Majority of the adverse health effects associated with aflatoxin such as development of Hepatocellular Carcinoma (HCC) are caused by exposure to subacute levels of aflatoxin in food products over a period (Meijer et al., 2021). A study from Africa Liver Consortium also revealed that HCC often develops much earlier in the younger population of African countries compared to other regions of the world (Yang et al., 2017).

The entry of these fungi not only compromises the self-defence of crops but negatively affects the crop's growth, yield, and market value (Kumar et al., 2021). Hence, there was a dire need to ascertain the level of aflatoxin contamination of some food staples in our local markets and seek for ways to reduce the level of consumers' exposure. There are several pieces of evidence of aflatoxin contamination of the selected food crops (groundnut, maize, and cowpea) in Nigeria (Adetunji et al., 2014; Ogara et al., 2017; Oyedele et al., 2017; Oyeka et al., 2019). Irrespective of numerous data on aflatoxin contamination of these food crops, only the study by Pessu et al. (2020) surveyed the prevalence of this contamination in two major markets in Port Harcourt, Nigeria. So, the present study surveyed microbial and aflatoxin contamination in groundnut (*Arachis hypogaea)*, maize (*Zea mays*), and cowpea (*Vigna unguiculata*) collected from Port Harcourt, Nigeria.

Materials and methods

Sampling

Ninety samples (30 samples in each group) of 250 g of maize, groundnut, and cowpea were purchased in April 2021 from five different vendors in six major markets in Port Harcourt, Nigeria (viz., Mile 1, Mile 3, Rumuokoro, Sangana, Borokiri, and Oil mill market) using random purposive sampling. The samples were collected in a zip lock bag and transported down to the food quality laboratory of Nigerian Stored Products Research Institute (NSPRI), Port Harcourt for analysis. The samples from each product were bulked and thoroughly mixed to get representative samples for analysis. Laboratory analysis, such as determination of Moisture Content (MC), microbial analysis, and determination of aflatoxin total, were carried out.

MC determination

MC of samples was determined according to the standard methods of Association of Official Analytical Chemist (AOAC International, 2016). Two g of each sample was weighed into clean and dried stainless steel moisture cans and oven dried at 105 $^{\circ}$C until a constant weight was attained. The samples were cooled in a desiccator and weighed using a sensitive analytical weighing balance.

Enumeration and isolation of bacteria

Total heterotrophic bacteria were determined by inoculating the surface of dried Nutrient Agar (NA) plates with aliquot (0.1 ml) of 10^{-6} dilutions in duplicates. The aliquot was evenly spread using sterile bent glass rod followed by incubation of the inoculated plates in the incubator at 37 $^{\circ}$C for 24 h. After incubation, colonies that grew on the respective plates were counted and used in enumerating the bacterial loads of the various food samples. Characterization of bacterial isolates was determined based on their morphological and cultural characteristics. Morphological characteristics adopted include color, shape, texture, size of colonies, and gram staining technique while the cultural characteristics adopted include sugar fermentation tests, Methyl Red (MR) test, Voges Proskauer (VP), indole, catalase, oxidase, and citrate tests. Identities of bacterial isolates were further authenticated by referencing their characteristics

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with those presented in the Bergy's manual of determinative biology (Prescott et al., 2011).

Enumeration and isolation of total heterotrophic fungi

Sabouraud Dextrose Agar (SDA) (Titan Biotech Ltd., India) was used to determine total heterotrophic fungi count. The spread plate technique was adopted (Prescott et al., 2011). An aliquot (0.1 ml) from 10^{-3} of the serially diluted samples was inoculated in duplicates onto surface of dried SDA plates and then spread evenly with a flame glass spreader. The plates were incubated at 25 °C for 72 h after which the colonies were counted, and the mean of the count recorded (Douglas and Robinson, 2018, 2019).

Identification of fungal isolates

Spores of the fungal isolates were picked using flamed inoculating pin, placed on a clean glass slide and gently spread with the inoculating pin and a drop of Lactophenol Cotton Blue (LPCB). The preparation was covered with a cover slip then observed with the aid of an electron microscope (OPTIKA, Italy). The presence or absence of septa in the mycelium, type of spore, presence of primary or secondary sterigmata, and other microscopic characteristics as well as cultural characteristics were used in the identification of the fungal isolates. These isolates were ascertained by comparison with a fungal atlas (Kidd et al., 2016).

Determination of total aflatoxins

Ridascreen aflatoxin total (Art. No R4,701) Enzyme-Linked Immunosorbent Assay (ELISA) kit was used for determination of total aflatoxins of the collected samples (R-Biopharm AG, Germany). Samples were prepared for the test procedure by grinding, extraction, filtration, and dilution. Two g each of the ground and homogenized sample was measured into 50 ml beaker and 10 ml of 70% methanol was added. The solution was homogenized at room temperature using an orbital shaker (ZD-9,556, USA). The extract was filtered using a filter paper (Whatman No. 1). The filtrates were diluted using 100 µl of the filtrate with 600 µl distilled water (seven dilution) and 50 µl of the diluted filtrate per well was used in the test. A wash buffer was prepared using the wash buffer salt Tween contained in the kit and according to the manufacturer's instructions. Prepared samples and 50 µl of the standard were added to separate wells and 50 µl of the conjugate and 50 µl of the antibody were added to each well. The contents of the wells were mixed gently and then incubated for 30 min at room temperature (20-25 °C). The liquid content of the wells was poured out and the microwell holder was tapped upside down against an absorbent paper to ensure complete removal of liquid from the wells. Each well was filled with 250 µl wash buffer and emptied again; this was repeated two more times. The substrate/chromogen (100 µl) was added to each well, mixed, and then incubated for 15 min at room temperature. Then the stop solution (100 µl) was added to each well and mixed gently by shaking the plate manually and absorbance was measured at 450 nm using a spectrophotometer (SPECTRUMLAB 22PC/REV A/08-99, UK) within 30 min after that the stop solution was added. Specific software, the RIDA SOFT Win.net (Art. No. Z9,996) was available for the evaluation of the RIDASCREEN enzyme immunoassays and it was used for evaluation of test results of the aflatoxin total.

Data analysis

Statistical analyses were performed using SPSS 20.0 by Analysis of Variance (ANOVA) and mean calculated and separated using Duncan's test when significant (p<0.05).

Results

Total aflatoxins

The results of the total aflatoxins content of the three crops (groundnut, maize, and cowpea) from the six markets are shown in Table 1. Among the three crops sampled, elevated levels of aflatoxin content were only recorded in groundnut and maize samples. The recorded values were above the acceptable World Health Organization (WHO) limits (0.5-15 µg/kg) for aflatoxin in legumes, except for cowpea which had values (0.069-11.86 µg/kg) within the acceptable limits. The highest aflatoxin level (95.6 µg/kg) was recorded in maize samples obtained from Rumuokoro market while the least one (0.057 µg/kg) was recorded in maize samples obtained from Mile 3 markets. The percentage mean values of total aflatoxins content of these crops above WHO acceptable limits are shown in Table 2.

MC

The MC of groundnut, maize, and cowpea samples bought from the six major markets are indicated in Table 3. Groundnut, maize, and cowpea MC ranged from 2.48-5.55, 9.00-11.25, and 9.50-12.48%, respectively. The highest MC (12.48%) was recorded in cowpea while the least MC (2.48%) was recorded in groundnut samples (Table 3). Low MC was recorded in groundnut samples across the markets.

Mean bacterial count

The bacteria isolated from the food crops were *Pseudomonas* sp., *Streptococcus* sp., *Clostridium* sp., *Bacillus*

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sp., *Staphylococcus* sp., *Proteus* sp., and *Escherichia coli*. *Pseudomonas* sp., *Streptococcus* sp., and *Clostridium* sp. were only isolated from groundnut samples while *Bacillus* sp., *Staphylococcus* sp., *Proteus* sp., and *E. coli* were isolated from the three samples. The most dominant bacterial isolates were *Bacillus* sp. and *Staphylococcus* sp. The results of the mean bacterial count (Colony Forming Unit (CFU)/g) of groundnut, maize, and cowpea are shown in Table 4. The highest bacterial count in the groundnut samples was recorded from samples bought from Sangana market (1.7×10^8 CFU/g), while the least bacterial count was recorded from groundnut samples bought from Borokiri market (1.0×10^8 CFU/g).

Mean fungal count

The result of the fungal count for the food crops are shown in Table 5. Seven fungal isolates included *A. flavus*, *A. niger*, *Penicillium* sp., *Rhizopus* sp., *Mucor* sp., yeast sp., and *Acremonium* sp. Their percentage of occurrence among the samples were as follows; 26.32% for *A. flavus* and *A. niger*, 15.79% for *Penicillium* sp., 10.53% for *Rhizopus* and *Mucor* sp., and 5.26% for yeast sp. and *Acremonium* sp. *A flavus* and *A. niger* were only isolated from groundnut and maize.

Entomological inspection of samples

It was observed that 50% of the maize samples were infested with *Sitophilus zeamais*, while 50% had oviposition. All the cowpeas were infected with *Callosobruchus maculatus*. There was no insect on the groundnut samples. Most of the markets treated the beans, so the insects were already dead.

Table 1: Aflatoxin contents (µg/kg) for the food crops purchased from six major markets in Port Harcourt, Nigeria

	Market					
	Mile 3	Mile 1	Sangana	Borokiri	Rumuokoro	Oil mill
Groundnut	10.79	13.40	33.79 *	11.71	24.29 *	68.84 *
Maize	0.057	<1.75	18.54 *	0.262	95.56 *	<1.75
Cowpea	0.069	11.86	0.250	<1.75	0.102	<1.75

*Indicates values above World Health Organization (WHO) recommended limits of <15 µg/kg

Table 2: Percentage mean value of total aflatoxin level of groundnut, maize, and cowpea purchased from six major markets in Port Harcourt, Nigeria

Food Crop	Total aflatoxin level (µg/kg)	Number above limits	% Mean value of food crop
Groundnut	>15	15	50 *
Maize	>15	10	33.33 *
Cowpea	>15	0	0

*Indicates percentage value above World Health Organization (WHO) recommended limits of <15 µg/kg

Table 3: Percentage of Moisture Content (MC) of groundnut, maize, and cowpea samples from six major markets in Port Harcourt, Nigeria

	Market					
	Mile 3	Mile 1	Sangana	Borokiri	Rumuokoro	Oil mill
Groundnut	2.48±0.044 [a]	4.53±0.033 [b]	5.22±0.017 [d]	4.52±0.044 [b]	5.02±0.044 [c]	5.55±0.029 [e]
Maize	11.25±0.029 [c]	9.00±0.058 [a]	10.00±0.058 [b]	10.42±0.022 [b]	10.15±0.029 [b]	10.00±0.058 [b]
Cowpea	10.10±0.029 [b]	10.48±0.044 [c]	12.48±0.044 [f]	9.50±0.029 [a]	10.75±0.029 [d]	11.00±0.029 [e]

Values expressed as Mean±SE (n=3). Values with different alphabets in a row are significantly (p=0.000) different while values with same alphabet in a row are not significantly (p=0.000) different.

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Table 4: Mean bacterial count (Colony Forming Unit (CFU)/g) of the various samples from six major markets in Port Harcourt, Nigeria

	Market					
	Mile 3	Mile 1	Sangana	Borokiri	Rumuokoro	Oil mill
Groundnut	1.0×10^8	1.6×10^8	1.7×10^8	0.7×10^8	1.3×10^8	1.1×10^8
Maize	0.8×10^8	0.3×10^8	0.6×10^8	1.3×10^8	0.7×10^8	0.6×10^8
Cowpea	0.7×10^8	1.6×10^8	1.6×10^8	0.9×10^8	1.6×10^8	1.9×10^8

Table 5: Mean fungal count (Colony Forming Unit/g) of the various samples from six major markets in Port Harcourt, Nigeria

	Market					
	Mile 3	Mile 1	Sangana	Borokiri	Rumuokoro	Oil mill
Groundnut	0.2×10^6	0.2×10^6	0.3×10^6	0.1×10^6	0.1×10^6	1.0×10^6
Maize	1.5×10^6	0.3×10^6	0.4×10^6	0.3×10^6	0.5×10^6	0.6×10^6
Cowpea	0.2×10^6	0.1×10^6	0.2×10^6	0.1×10^6	0.1×10^6	0.5×10^6

Discussion

The high aflatoxin levels recorded in groundnut purchased from Sangana, Rumuokoro, and Oil mill markets concur with the prevalence data collated by Partnership for Aflatoxin Control in Africa (PACA) in 2013 on aflatoxin contamination in groundnuts. In their report, high proportions of raw groundnut samples and its products tested positive for *Aspergillus* infection and aflatoxin contamination before and after harvest.

Nuts have been adjudged to be one of the most susceptible agricultural commodities to aflatoxin contamination (Anthony et al., 2012). The high susceptibility of nuts and their products to aflatoxin contamination can be attributed to their high protein composition (Adetunji et al., 2020). Groundnut and its products have a long-standing history of aflatoxin contamination in Nigeria and have been widely reported in previous studies. In a study by Bankole and Adebanjo (2003), the aflatoxin level of groundnut cake from Nigeria was as high as 20-455 ppb, considered to be unfit for consumption. Bankole and Adebanjo (2003) also reported that out of 106 samples of roasted groundnuts, 68 were contaminated with aflatoxin B_1 in the range of 5-165 ppb, while 28 had aflatoxin B_2 in the range of 6-26 ppb, 12 out of the total number of samples had aflatoxin G1 in the range of 5-20 ppb while only 3 contained aflatoxin G2 in the range of 7-10 ppb.

High aflatoxin levels recorded in groundnut may be because of the time of the year (April) in which the survey was conducted. It has been reported that aflatoxin levels in agricultural commodities may have a direct correlation with the season of the year (Anthony et al., 2012; Mutegi et al., 2009). In another report by Kamika and Takoy (2011), highly contaminated agricultural commodities were purchased during the rainy season with a 90% frequency at 12-939 ppb compared to the dry season with 53.2% frequency and a range of 15-390 ppb. Post-harvest handling of agricultural produce also plays a huge role in the extent of aflatoxin contamination (Adeyeye, 2016; Chauhan et al., 2008). This justifies the significant differences recorded in the aflatoxin content of groundnut samples across the six markets. The result of this study also concurs with data presented by Pessu et al. (2020) who reported elevated levels of aflatoxin contamination in groundnut samples obtained from the Port Harcourt, Nigeria.

The percentage mean value of aflatoxin in maize (33.33%) recorded in this study concurs with the findings of Pessu et al. (2020) who recorded high levels of aflatoxin (>4 ppb) in maize samples purchased from two markets in Port Harcourt. High aflatoxin levels in maize have also been widely reported by several other studies in Nigeria (Atehnkeng et al., 2008; Ibrahim et al., 2021). About 90% of maize samples in East Africa contain high levels of aflatoxin while it is as high as 99% in some parts of West Africa (Rodrigues et al., 2011; Shephard, 2004). Although, high levels of aflatoxin were recorded in maize samples (Table 1), 20 out of 30 analysed samples had very low values within WHO acceptable limits (<15 µ/kg). Good post-harvest practices may be responsible for this (Adeyeye, 2016).

Low levels of aflatoxin contamination in cowpea recorded in this study concur with previous findings (Ibrahim et al., 2021; Pessu et al., 2020). Generally, cowpeas are less susceptible to aflatoxin contamination compared to maize and groundnut (Houssou et al., 2009). However, the result disagrees with the findings of Ogungbemile et al. (2020) who detected aflatoxin contamination in all the

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cowpea samples from Ibadan, Nigeria. An earlier report by Seenappa et al. (1983) found aflatoxin contamination in stored cowpea in Tanzania. Houssou et al. (2009) also detected aflatoxin B_1 in samples of cowpea seeds collected after few months of storage in the republic of Benin, West Africa. Climatic factors, storage conditions, and post-harvest handling may have contributed to these differences in aflatoxin contamination (Adeyeye, 2016; Chauhan et al., 2008).

The MC of food crops gives a preview into the extent of possible fungal growth inhibition and durability of crops during storage. High MC in food crops (12-14%) is associated with fungal infection and aflatoxin contamination (Chigoziri and Temitope, 2020; Wu et al., 2011). The MC of the food crops in the present study revealed otherwise (Table 3). This suggests that the preceding premise on the correlation between high MC in crops and fungal infection may be relative. Groundnut samples which recorded the highest aflatoxin contamination had the least MC range (2.48-5.55%) compared to maize and cowpea (9.00-11.25% and 9.50-12.48%, respectively). However, Atanda et al. (2013) and Ok et al. (2014) reported that there is a tendency for *A. flavus* to grow on grains with lower MC. This result concurs with the findings of Ibrahim et al. (2021) who recorded low MC (6.02 and 9.07%) in the aflatoxin contaminated groundnut and maize samples.

Microorganisms are ubiquitous (Prescott et al., 2011), thus the ease at which they are freely transported can lead to contamination of food samples. Previous studies have reported that microbial contamination of food samples is influenced by a range of factors which include handling, processing, storage, and transportation (Hammond et al., 2015; Wang et al., 2018). *Bacillus* sp., *Clostridium* sp., and *Staphylococcus* sp. isolated in this study have been associated with food-borne infections and intoxications (Kharel et al., 2016). *Staphylococcus* sp. are part of the normal flora of the skin (Prescott et al., 2011) and the presence of *Staphylococcus* sp. in food samples arises due to contamination from handlers or packaging materials used in conveying the food samples for sale (Ho et al., 2015).

A. flavus and *A. niger* isolated from maize and groundnut samples only may be linked to the aflatoxin contamination of these two crops as presented in this study. The results are in line with the reports of Ibrahim et al. (2021) who isolated the two fungi from groundnut and maize samples ladened with aflatoxin. Chigoziri and Temitope (2020) also isolated same from groundnut samples which contained aflatoxin B_1. *A. flavus* is notorious for aflatoxin production (Rodrigues et al., 2007). It produces a variety of enzymes which facilitate the growth of fungi on stored agricultural commodities such as corn, peanuts, soybeans, and groundnuts (CDC, 2004). When ingested, aflatoxins are converted to aflatoxin-8, 9-epoxide metabolite in the liver which may lead to many toxic effects in the human system (Eaton and Gallagher, 1994). The absence of these two fungi in cowpea is relatable. This validates the absence of aflatoxin contamination on this crop as recorded in this study.

Dead insects (*Callosobruchus maculatus*) discovered on the cowpea samples were due to the application of insecticides which are synthetic, organophosphate or pyrethroid compounds notorious for causing accidental poisoning and death (Ezechukwu and Szlatcheka, 2001; Ikpesu and Ariyo, 2013; Nwaubani et al., 2014). The distribution of this insect as major insect pest has also been reported by Nwosu et al. (2020) who stated that cowpea infestation by *C. maculatus* during storage exposes farmers and consumers to degrees of food shocks. Ashamo et al. (2021) also reported that *C. maculatus* poses a major constraint in the commercialization and post-harvest storage of cowpea. Additionally, *C. maculatus* causes more than 60% seed damage during storage (Ileke, 2015, 2019; Ileke et al., 2021; Oni, 2014). *Sitophilus zeamais* which was identified on the maize samples has also been reported by Nwosu et al. (2020) to proliferate during the storage of this crop thereby causing damage to the grains. Mechanical damages caused by these insects also facilitate the entrance of *A. flavus* (Jeyaramraja et al., 2018; Kinyungu, 2019). Good post-harvest practices which ensure proper storage of these crops under appropriate conditions would reduce the rate of insect infestation on these crops, subsequently reducing post-harvest loss of these crops.

Conclusion

This study revealed the health risk exposure of consumers of the assayed staples in Port Harcourt (Nigeria), especially groundnut which had very high aflatoxin levels in most of the markets. The high aflatoxin levels in these food crops raise concerns for the susceptibility of consumers to aflatoxin related diseases as this substance is a known carcinogen. Stakeholders in the food industry and agricultural sector should review the food value chain from farm to folk and identify critical control points in managing the situation.

Author contributions

I.U.N designed and supervised the work; M.E.I. and E.F.N. carried out the microbial analysis; E.A.A., C.H.O., and O.O.O. carried out the aflatoxin analysis and MC determination; M.K.A. inspected the samples for entomological parameters and carried out the data analysis; C.H.O. also did the literature search and wrote the

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manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest.

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