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Fungal diversity and mycotoxin contamination in dried fish products in Zhanjiang market, China

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ABSTRACT

Dried fish are important dietary protein and income sources in Zhanjiang, China. Mycotoxins produced by pathogenic fungi that contaminate fish during processing can cause considerable hazard to consumer health. This study reports fungal diversity, total fungal counts and mycotoxin contamination of dried fish sold at the seafood market in Zhanjiang. Seven dried fish products (*Hemibarbus maculatus, Pseudosciaena crocea, Lutjanus erythopterus, Thunnus thynnus, Scomberomorus niphonius, Eleutheronema tetradactylum, Trichiurus lepturus*, n = 10) from seven retailers were analyzed for contaminated fungal species, occurrence frequency and residues analysis of four mycotoxins. Using potato dextrose agar (PDA) plate purification, morphology observation, internal transcribed spacer (ITS) sequence analysis, 25 fungal strains representing 12 genera from dried fish were systematically isolated and identified. Three dominant genera in dried fish were *Fusarium* sp. (80.4%), *Penicillium* sp. (70.7%) and *Aspergillus* sp.(63.9%). Other fungal genera were *Neoscytalidium* sp.(38.1%), *Cutaneotrichosporon* sp. (38.1%), *Trichoderma* sp.(20.3%), *Naganishia* sp.(15.3%), *Kodamaea* sp. (10.8%), *Phialemoniopsis* sp.(9.2%), *Nigrospora* sp.(7.3%), *Ceriporia* sp.(6.3%), *Phellinus* sp.(4.5%). *Aspergillus flavus* contamination was the higher and ranged from 1.10×10^3 to 2.40×10^4 cfu/g. The mean fungal contamination of other fungal species in dried fish ranged from 1.07×10^2 to 4.58×10^4 cfu/g. The total fungal counts of *Fusarium* sp. ranged from 1.09×10^2 to 2.11×10^4 cfu/g, but the occurrence frequency is relatively high. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses showed that mycotoxin residues were present in 12 out of the 25 dried fish tested. Aflatoxin B_1 (AFB_1) was the most frequently detected and the concentration ranged from 0.03 to 3.52 μg/kg. T-2 toxin (T-2), ochratoxin A (OTA), and deoxynivalenol (DON) concentrations ranged from 0.21 to 1.53, 0.03–2.21 and 0.71 μg/kg respectively. High occurrence of fungal populations and mycotoxins in dried fish sold in the Zhanjiang market pose a potential threat to consumer health. It is recommended that in future, advanced processing methods and controlled storage condition need to be used to minimize and if possible eliminate fungal contamination during dried fish processing.

1. Introduction

Fish is one part of the important animal protein sources in the tropics and is exceptionally rich in calcium (Ca) and phosphorus (P) and β vitamins (Oluwaniyi &;Dosumu, 2009). Consumption of fish and fish products is popular especially in coastal areas. However, fresh fish is perishable and are hence processed to improve shelf life. Currently, chilling, freezing, salting, canning, drying and smoking, are the most commonly used techniques used for effective preservation (Kumolu--Johnson, Aladetohun & Dimele, 2010). Among these drying is the most

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popular. In India, about 17% of the total seafood catch is dried (Jeya, Vijayalakshmi, & Jeyasekaran, 2003). In the southern China, especially in Zhanjiang (Fig. 1), the annual fresh fish production is > 100, 000 kg. Salted-fish drying is the traditional method for seafood preservation in coastal areas and is very popular with the consumers. However, drying fish does not completely inhibit microbial growth. The microbial presence lowers the quality of fish products.

In most instances, fresh seawater fish are salted and dried in the sun in preference to professional food processing industry. This can lead to food safety issues because the drying conditions cannot be easily regulated with some fishermen prefer to dry them on streets or in open balconies of houses as practiced in many Asian and African countries (Akwuobu, Antiev, & Ofukwu, 2019; Gonkowski, Obremski, Makowska, & Rytel, 2018; Sam, Jeyasanta, & Edward, 2015). The quality of dry fish prepared outdoor by open sun drying is compromised by the bacterial, fungal, insect, and rodent contamination, and also by the unhygienic handling, and poor quality of salt and water used for fish processing (Sugumar, Jawahar, & Jayachandran, 1995). Several studies have reported the decline in dried fish quality of affected by fungal growth (Hassan, Hassan, El Shafei, El Ahl, & El-Dayem, 2011; Begum, Akter, Ahmed, & Md, 2011; Adeyeyea, 2016; Sa'adatu, Nura, & Muhammad, 2019). Thus, microbial contamination affects the meat quality by alteration of flavour, texture and loss of nutrients and also food safety problems (Singapurwa, Suprapta, Gunam, & Khalimi, 2018), all of which can result in a huge economic loss to the industry.

According to the reports, a range of fungal species isolated from dried fish can accelerate the deterioration of dried fish (Atapattu, Sama, Ajeewa, 1990; Zachariasova et al., 2014; Dègnon, Atrevy, Adjou, Ahoussi, & Soumanou, 2018). Common fungal genera that contaminate dried fish were *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp., and *Fusarium* sp. (Olajuyigbe et al., 2014; Jimoh et al., 2014). Ayeloja, George, Jimoh, Shittu, and Abdulsalami (2018) reported the growth of *A. flavus*, *F. oxysporum*, *Ceotrichium albidium*, *Penicillium* sp, and *Trrichoderma* sp. in smoked fish sold in Ibadan. Fagbohun and Lawal investigated isolated *A. flavus* and *Penicillium* sp. (both about 27% each) > *Rhizopus* sp., and *Fusarium* sp. fungal species in smoked dried fish products in different markets in Nigeria. (Fagbohun & Lawal, 2015). In Kenya's Gucha South district market, *A. niger* (17.57%) and *Rhizopus* sp. (29.73%) were the highest fungal species recovered from sun dried fish (Nyamwaka, Nyamache, & Maingi, 2017). To our knowledge, there are only a few reports on contaminated fungal species in dried fish in coastal areas of China. Therefore, the investigation of fungal diversity in dried fish provides an important data for risk assessment of dried seafood safety.

Mycotoxins are the secondary metabolites produced by various filamentous fungal species and the most common are aflatoxins (AFs), ochratoxins (OTs), *Fusarium* mycotoxins (e.g. deoxynivalenol (DON), T-2 toxin, zearalenone (ZEN)) and fumonisins (FBs) (Adekoya, Obadina, Phoku, Nwinyi, & Njobeh, 2017; Zachariasova et al., 2014). Mycotoxin concentrations vary depending on season, substrate and storage conditions. They are generally stable and difficult to eliminate from the food chain (Omotayo, Omotayo, Mwanza, & Babalola, 2019). Due to the inadequate control of temperature and humidity during storage, dried fish products are highly prone to mycotoxin contamination. Much mycotoxin research have been conducted in some tropical regions in Africa (Akinyem et al., 2011; Fagbohun & Lawal, 2015) and India (Sam et al., 2015), Fagbohun & Lawal (2015) detected 2.73–4.03 μg/kg AFB_1 and 2.01–3.53 μg/kg AFG_1 residues in 50 smoked dried fish samples randomly purchased from the main market of Ado-Ekiti, Nigeria. Gonkowski et al. (2018) reported 100% ZEN contamination of sun-dried fish in Zambia. Thus humans could be exposed to multiple potentially hazardous mycotoxins when dried fish products are consumed. Tolosa, Barba, Font, and Ferrer (2019) have found small amounts of fusarenon-X and enniatin B were residued in smoked fish during processing.

In China, Sun et al. (2015) reported that 3.5–317.3 μg/kg of ZEN and 1.9 μg/kg OTA residues in dried seafood products sold in Shanghai. Zhanjiang is located in the southern part of China. It is usually hot/warm and humid which is ideal for fungal growth and mycotoxin production. but no studies have been carried out in relation to fungal and mycotoxin contamination in dried fish products obtained from southern China markets. This study was conducted to investigate the diversity, occurrence frequency of contaminated fungi species and analyse quantificationally of mycotoxin concentration of dried fish products sold in seafood market in Zhanjiang.

Fig. 1. Geographical location of Zhanjiang, China.

2. Materials and methods

2.1. Sampling collection and preparation

A total of 70 samples including seven dried fish species (*Hemibarbus maculatus, Pseudosciaena crocea, Lutjanus erythopterus, Thunnus thynnus, Scomberomorus niphonius, Eleutheronema tetradactylum, Trichiurus lepturus*, n = 10) were randomly sampled from seafood markets in Zhanjiang, China. Dried fish products were transferred into individual polythene bags, sealed, labeled and transported to the Guangdong Ocean University microbiology laboratory in Zhanjiang. The samples were stored at ambient temperature until analysis.

2.2. Mycological analysis

The 25 g dried fish were cut into small cubes and homogenized with 225 mL of sterile distilled water. Following a ten-fold serial dilution, 1 mL diluent was spread on the Potato Dextrose Agar (PDA) medium with 40 μg/mL chloramphenicol. Different morphology, color, size of the colonies were enumerated (cfu/g) respectively after 5 days of incubation at 28 °C.

2.3. Fungal isolation and purification

The 100 μL of the above mentioned diluent (in section 2.2) of each sample was spread on sterile PDA medium and incubated at 28 °C for 4 d. Morphologically distinct colonies were sub cultured on fresh media to obtain pure isolates and further maintained at 4 °C on PDA medium. After 4 d of incubation, different fungal species were distinguished through colony morphology, color, size and texture. Pure isolates were numbered and stored at −20 °C for further identification.

2.4. DNA extraction, polymerase chain reaction and sequencing

Molecular identification was conducted on the basis of nucleotide sequence of DNA. The DNA of each fungus was extracted using a kit (Ezup Column Fungi Genomic DNA Purification Kit, Sangon Biotech Co.,

Ltd., China). The ribosomal rRNA gene was amplified using universal fungal primers: internal transcribed spacer 1 (ITS1: 50-TCC GTA GGT GAA CCT GCGG-30), and internal transcribed spacer 4 (ITS4: 50-TCC TCC GCT TAT TGA TAT GC-30). The PCR reaction mixture was prepared using EF-Taq as follows: 10 × EF-Taq buffer 2.5 μL, 10 mM dNTP (T) 0.5 μL, each primer (10 pmol) 1.0 μL, Taq DNA polymerase (0.6 U) 0.5 μL, DNA template 1.0 μL, double distilled water (up to 25 μL). PCR amplification was performed in a thermal cycler (LongGene Thermal Cycler, LongGene Scientific Instruments Co., Ltd., China) with the following cycling parameters: one cycle of denaturation at 95 °C for 15 s followed by 30 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 15 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min. To evaluate the quality of the amplicons, 5 μL PCR products were analyzed by electrophoresis in 1% standard agarose gel (using a DNA ladder). Gels were electrophoresed at 100 V for 30 min and photographed using a UV transilluminator and Gel Smart system software. Amplification products were sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing. The sequence was compared with other ITS rDNA gene sequences obtained from the NCBI GenBank database. The resulting alignment was loaded and analyzed with the phylogenetic analysis program MEGA version 7.0. The best model was estimated for each dataset considering the Bayesian information criterion (BIC) and the Akaike information criterion (AIC).

2.5. Dried fish: mycotoxin analysis

2.5.1. Mycotoxin extraction

Two gram of dried fish was added with 10 mL of acetonitrile/water (v: v, 85/15), homogenized (IKAT25, Staufen, Germany) for 1 min (10,000 r/min). The mixed solution was subjected to ultrasound assisted extraction (UAE) (PS-30 A, power: 180 W, frequency: 40 kHz, Ruimi Instruments Co., Changzhou, China) for 60 min at 20 °C, then centrifuged at 4500×*g* for 10 min. This extraction process was repeated twice and the supernatants were combined. The supernatants were mixed and subsequently evaporated to dryness using a gentle nitrogen stream at 50 °C, then redissolved in 1 mL of methanol/water (30/70, v/v) with 0.1% formic acid. The solution was ultrasonically irradiated for 3 min and mixed by vortexing (XW-80 A, Haimen, China) for 1 min. The solution was then filtered through a 0.22-μm filter (diameter-25 mm, polyamide 6, organic phase, Tianjin, China) and stored at −20 °C until LC-MS/MS analysis.

2.5.2. LC-MS/MS analysis

Toxin analysis was performed on a Thermo Scientific Surveyor HPLC system that comprised a Surveyor MS Pump Plus, an on-line degasser, and a Surveyor Autosampler Plus coupled with a Thermo TSQ Quantum Access tandem mass spectrometer equipped with an electrospray ionization (ESI) source (Table 1).

(Massachusetts, USA). The separation was performed at 35 °C using a Hypersil GOLD column (5 μm, 100 mm × 2.1 mm) (Thermo Scientific, CA, USA) with a flow rate of 0.25 mL/min. The injection volume was 5 μL. The mobile phase consisted of methanol (A) and water containing 5 mM ammonium acetate 0.1% formic acid (B) with the gradient elution program as follows: 0 min 30% A, 3.0 min 90% A, 5 min 90% A, from 5.1 min to 8 min 30% A and hold on for a further 2 min for re-equilibration, giving a total run time of 10 min.

MS/MS detection was carried out using a triple quadruple mass spectrometer, coupled with an electrospray ionization source operated in both positive (ESI+) mode (Shimadzu, Kyoto, Japan). The ionization source parameters were set as follows: spray voltage: 4500 V; sheath gas pressure: 35 au; ion sweep gas pressure: 0 au; auxiliary gas pressure: 15 au; capillary temperature: 350 °C; tube lens offset: 118 V; skimmer offset: 0; collision energy: 1.5 eV; and collision pressure: 1.5 mTorr. Quantitation was performed in multiple reaction monitoring (MRM) mode and the conditions were optimized for each mycotoxin during infusion. Through optimization, the limit of detection (LOD) of AFB_1, T-2, OTA and DON were 0.1, 0.1, 0.1 and 1.0 μg/kg, respectively, limit of quantification (LOQ) were 0.3, 0.3, 0.5, 3.0 μg/kg, respectively. The recovery of four mycotoxin in dried fish ranged from 90.1 to 103.8%.

Table 1
Optimal precursor and product ions of analytes of the mycotoxins with the respective collision energy (eV) values used in MS/MS analysis.

Toxin	Precursor ion (m/z)	Product ions (m/z)	CE[b](eV)	Retention time (min)
AFB_1	313.0 $[M+H]^+$	213.0	44	5.90
		241.0[a]	36	
T-2	484.3 $[M+NH_4]^+$	185.1[a]	27	6.30
		215.1	25	
OTA	402.0 $[M-H]^+$	238.1	27	4.91
		358.1[a]	15	
DON	295.0 $[M-H]^+$	203.1	16	5.25
		249.1[a]	12	

[a] Quantitative ion.
[b] CE: collision energy.

2.6. Data analysis

Sequences were further analyzed by BLAST from the National Center of Biotechnology Information (NCBI) website. The data were analyzed again by aligning the sequence though using MEGA version 7.0 program.

3. Results and discussion

3.1. Isolation and diversity of contaminated fungi

Twenty-five fungal isolates with different colony morphology were isolated from seven types of dried fish products (Fig. 2). Twenty-two isolated colonies were filamentous fungi and 3 colonies (ZJC23, ZJC24, ZJC25) showed a yeast-like morphology in PDA agar. By analysis, In the present study the contaminating fungal mycobiota form seven dried fish products was identified. Isolates from 12 fungal genera were identified. Three dominant genera were *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. This finding is consistent with Nyamwaka, Nyamache, & Maingi (2017) who reported that the most common fungal genera grew in sun dried fish sold in market. *Aspergillus* sp. is the most common fungus in dried fish reported by many research (Hassan et al., 2011; Olajuyigbe et al., 2014; Jimoh et al., 2014; Wogu & Iyayi, 2011). Except common fungal genera, several uncommon fungal genera such as *Neoscytalidium* sp. *Ceriporia* sp., *Nigrospora* sp., *Cutaneotrichosporon* sp., *Naganishia* sp. *and Phialemoniopsis* sp. were isolated in the dried fish products. Previous studies have shown that fungal contamination of sun dried fish vary can be regional specific with *Saccharomyces* sp., *Mucor* sp., *Rhodotorula* sp., *Schizosaccharomyce* sp., *Acremonium* sp., *Rhizopus* sp., *Absidia* sp., *Aureobasidium* sp., *Trichoderma* sp., *Cladosporium* sp., *Alternaria* sp., and *Candida* sp. isolation in different regions (Olajuyigbe et al., 2014; J; ob, Agina, & Dapiya, 2016). In addition, the uncommon fungi species *Candida tropicalis*, *Candida stellatoidea*, *Microsporum audunii*, *Trichophyton rubrum* were isolated from smoked-dried fish sold at Nigeria (Fatima, Idris, & Gloria, 2016). In this study, we observed that filamentous fungi contamination of dried fish was more prevalent than yeast-like fungi, which may be due to its stronger reproduction and diffusion ability. Spore production in filamentous fungi make it easy to adhere and survive in high protein nutrient sources such as dried fish. The species of contaminated fungi required further molecular identification. Agarose electrophoresis results showed successful PCR amplification of 25 strains (Fig. 3).

3.2. Genetic identification of contaminated fungi

Amplified fungal sequences were used as BLAST queries against the

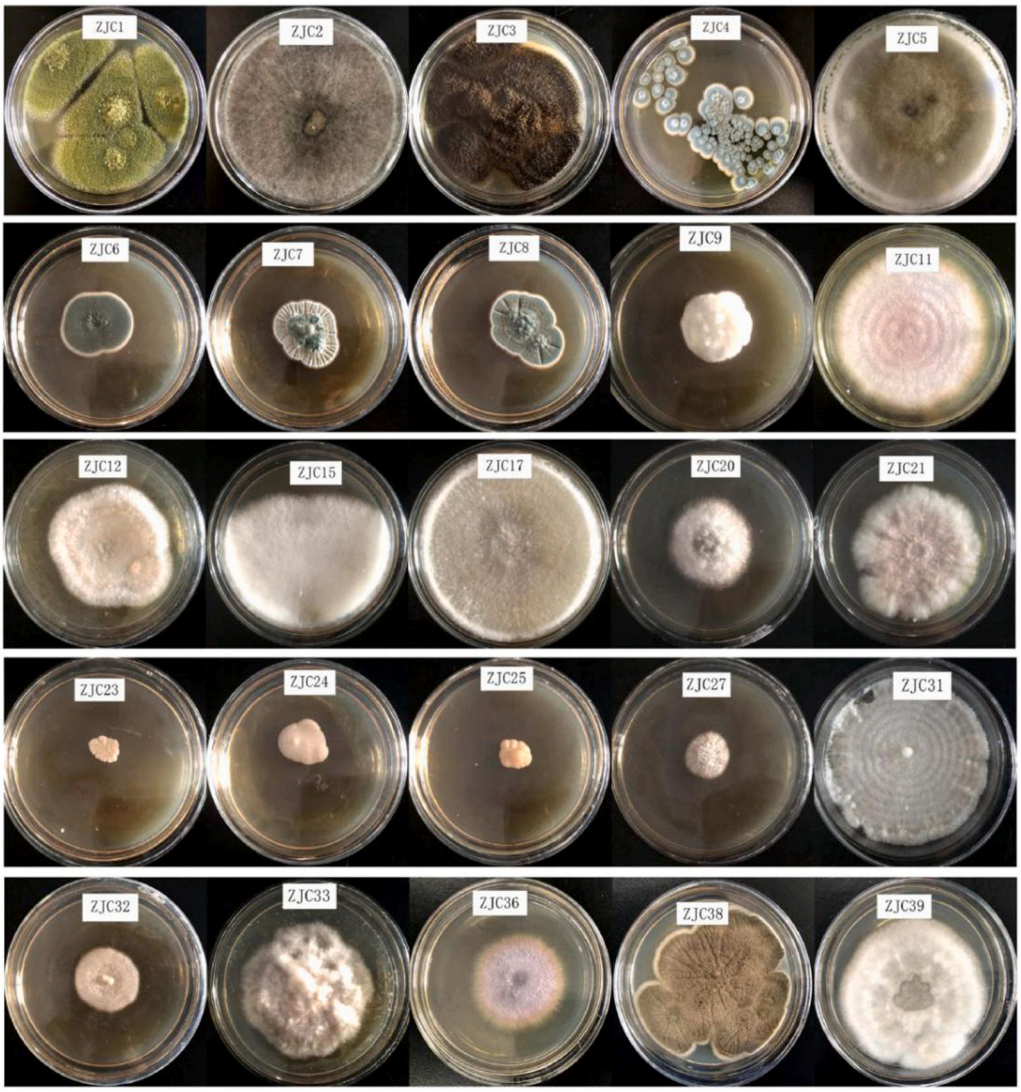

Fig. 2. Colony morphology of 25 fungal isolates from seven dried fish products sold at the Zhanjiang market, China.

NCBI database. It shows the fungi isolated from dried fish samples had 97–100% similarity compared with closely related fungi in the GenBank. Through BLAST alignment analysis, the molecular identification results of 25 strains are shown in Fig.3.

According to genetic identification, three types of *Aspergillus* sp. isolated from dried fish were *A. flavus, A. fumigatus* and *A. niger (see* Table 2*)*. *A. flavus* is the most common of *Aspergillus* fungi that has been reported to contaminate most of smoked and sun dried fish (Atef, Manal, Howayda, Rasha, & Abdel-Dayem, 2011; Akinyemi, Adejola, Obasa, & Ezeri, 2011; Olajuyigbe et al., 2014; Osibona, Gunyebi, & Samuel, 2018). *Aspergillus* sp. can produce a large number of conidia and the high moisture promotes the germination of its spores, which increases the risk of fungal production in dried fish. In addition to the above 3 *Aspergillus* species, *A. tamari*, *A. sydowii*, *A. versicolor*,*A. fumigatus*,

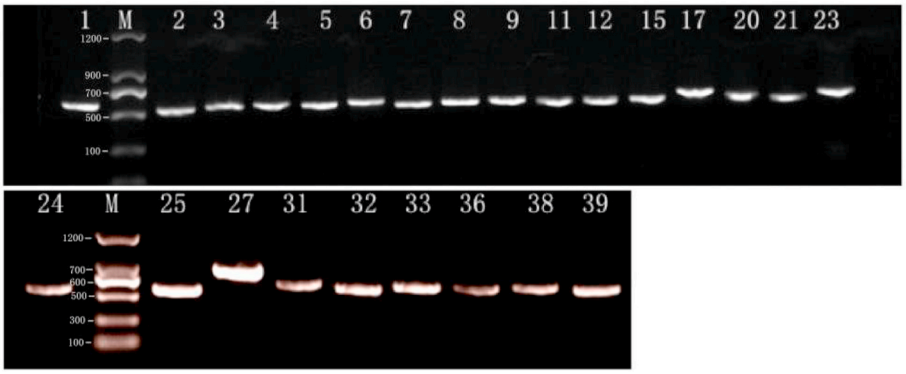

Fig. 3. PCR amplifications of rRNA gene with ITS 1 and ITS 4 primers in 25 isolates (different numbers represent different isolates, M:DNA ladder).

A. aculeatus, *A. tubingensis*, *A. tereus*, *A. ochraceus* and *A. wentii* have also been reported in dried salted fish (Fafioye, Efuntoye, & Osho, 2001; Adebayo-Tayo, Onilude, & Ukpe, 2008; Osibona, Gunyebi, & Samuel, 2018; Singapurwa et al., 2018). Aflatoxin-producing fungi isolation means the potential contamination with mycotoxins. Exposure to aflatoxins can cause chronic health disorders including hepatitis B and hepatocellular carcinoma, which seriously threaten human health (Liu, Chang, Marsh, & Wu, 2012).

Penicillium citrinum was detected in the dried fish samples and also reported in dried fish samples sold in Giza, Eygpt (Hassan et al., 2011). *Penicillium oxalicum* and *P. christenseniae* were initially found in dried fish and this may have been due to the higher water activity and storage temperature conditions in Zhanjiang, as such conditions are most suitable for *Penicillium* sp. growth. Nine isolates from 7 *Fusarium* species were detected in samples which reflects the diversity of *Fusarium* contamination of dried fish products. These results are in line with those reported by Fafioye, Efuntoye, and Osho (2002) and Nyamwaka, Nyamache, & Maingi (2017), who reported that *Fusarium* contamination of smoke-dried fish in Nigeria and sun dried fish in Kenya, respectively. Among the three dominant fungal genera, *Trichoderma* sp. and *Saccharomyces* sp. are the most common fungi isolated from dried fish products (Wogu & Iyayi, 2011; Ayeloja et al., 2018). It is interesting that *Rhizopus* sp. and *Mucor* sp. are the fungal genera widely isolated from dried fish (Fafioye et al., 2002; Oku & Amakoromo, 2013; Akwuobu et al., 2019) but we did not detect them in our samples. *Ceriporia lacerata* and *Phellinus noxious* are the rot fungi commonly present in decayed plants (Sahashi et al., 2015; Wang, Chu, Wu, Zhao, & Xu, 2017) and basidiospores of *P. noxious* are highly pathogenic and can cause serious brown root rot disease (Hsiao, Hung, & Sun, 2019). The isolation of two rot fungi in the dried fish samples suggest environmental contamination of dried fish by rot fungi during drying of fish on the wood. *Phialemoniopsis* sp. is a pathomycete widely distributed in the air, industrial water, plant material, river water, sewage, and soil (Tsang et al., 2014). It has been reported to cause serious skin and soft tissue infections in humans (Desoubeaux et al., 2014; Perdomo et al., 2013). In our study, the presence of 25 fungal isolates from dried fish samples could be due to the unclean processing environment and/or disregard to conditions of strict temperature and humidity control.

3.3. Mycological survey

Table 3 shows that dried fish samples sold in the market were contaminated with a range of different fungal species with total counts ranged from $1.07 \pm 0.04 \times 10^2$–$0.4.58 \pm 1.37 \times 10^4$ cfu/g. Generally, it could be assumed that the dried fish sold by different retailers would be contaminated with different fungal level. The *A. flavus* contamination was detected in seven types of dried fish products with relatively high values ranging from $1.10 \pm 0.06 \times 10^3$ to $2.40 \pm 0.32 \times 10^4$ cfu/g. *A. niger* counts varied from $1.25 \pm 0.22 \times 10^2$ to $3.10 \pm 0.38 \times 10^3$ cfu/g isolated from four dried fish species while *A. fumigatus* was $7.21 \pm 1.76 \times 10^2$ cfu/g detected in only *S. niphonius* (Fig. 4). The total fungal counts was consistent with previous reports. Job, Agina & Dapiya (2016) reported the *A. flavus* contamination in smoked-dried fish was $1.00 \pm 0.42 \times 10^3$–$9.00 \pm 3.82 \times 10^3$ cfu/g. The contamination frequency of *Aspergillus* sp. in dried fish was 63.9%, significantly higher than in dried fish sold in North-Central Nigeria open markets (28.6%) (Akwuobu et al., 2019), but lower than in dried fish sold in Zambia (88%) (Kachapulula, Akello, Bandyopdhyay, & Cotty, 2018). Thus the fungal contamination significantly varied between regions and countries. Nyamwaka, Nyamache & Maingi (2017) reported a contamination frequency 17.6% of *A. niger*, 9.5% of *A. flavus*, 10.8% of *A. fumigatus*. The species most frequently isolated from dried fish samples is *A. flavus* and this is an indication of its ubiquitous nature (Fafioye et al., 2002; Osibona, Gunyebi, & Samuel, 2018). This is mostly related to the strong spore reproduction and mycelium diffusion ability of *Aspergillus* sp. (Wang, Dijksterhuis, Wyatt, Wösten, & Bleichrodt, 2015). High frequency of *Aspergillus* sp. contamination carries a potential risk of aflatoxin production, which seriously endangers human health.

Penicillium sp. is a common contaminating genus isolated from dried fish. In this study, a high frequency of occurrence of *P. citrinum*, *P. christenseniae* and *P. oxalicum* ranging from $1.23 \pm 0.04 \times 10^3$–$4.58 \pm 1.37 \times 10^4$, $2.10 \pm 1.00 \times 10^3$–$3.69 \pm 1.72 \times 10^4$ and $2.17 \pm 0.24 \times$

Table 2
Identification of fungal isolates recovered from different dried fish types by sequencing of internal transcribed spacer 1 and 4 (ITS1 and ITS 4) and the region of ITS rRNA gene compared with sequences listed in the GenBank for similar species.

Fungi isolated from dried fish				Fungal gene bank		Similarity (%)
ZJC No.	Fungi	No. Of nucleotides		Accession No. In Bank	Fungi	
		With ITS1 primer	With ITS4 primer			
1	*Aspergillus flavus*	574	597	MH591448.1	*Aspergillus flavus*	99
2	*Neoscytalidium dimidiatum*	553	553	KX464231.1	*Neoscytalidium hyalinum*	99
3	*Aspergillus niger*	551	542	FJ431207.1	*Aspergillus niger*	99
4	*Penicillium citrinum*	545	538	MG733753.1	*Penicillium citrinum*	100
5	*Trichoderma harzianum*	557	557	MF871551.1	*Trichoderma harzianum*	99
6	*Penicillium oxalicum*	533	530	MG733738.1	*Penicillium oxalicum*	97
7	*Penicillium christenseniae*	577	576	MK267451.1	*Penicillium expansum*	98
8	*Penicillium christenseniae*	553	551	MK267451.1	*Penicillium expansum*	98
9	*Fusarium equiseti*	544	542	MH578583.1	*Fusarium equiseti*	99
11	*Fusarium oxysporum*	529	523	MH879861.1	*Fusarium oxysporum*	98
12	*Fusarium equiseti*	546	539	MH581383.1	*Fusarium equiseti*	99
15	*Phellinus noxious*	573	573	HQ400698.1	*Phellinus noxious*	98
17	*Ceriporia lacerata*	540	538	JX623917.1	*Emmia lacerata*	99
20	*Nigrospora sphaerica*	545	544	MG733725.1	*Nigrospora sphaerica*	97
21	*Fusarium chlamydosporum*	550	561	MG250446.1	*Fusarium chlamydosporum*	98
23	*Kodamaea ohmeri*	586	589	KY103876.1	*Kodamaea ohmeri*	99
24	*Cutaneotrichosporon mucoides*	568	564	KY103029.1	*Cutaneotrichosporon arboriformis*	99
25	*Naganishia diffluens*	555	541	KY104326.1	*Naganishia diffluens*	99
27	*Phialemoniopsis curvata*	577	576	NR132076.1	*Phialemoniopsis curvata*	99
31	*Fusarium incarnatum*	523	512	MH290471.1	*Fusarium incarnatum*	99
32	*Fusarium avenaceum*	554	546	MF509747.1	*Fusarium acuminatum*	98
33	*Fusarium nelsonii*	583	575	GQ505434.1	*Fusarium nelsonii*	99
36	*Fusarium verticillioides*	551	559	JH654505.1	*Fusarium verticillioides*	99
38	*Aspergillus fumigatus*	538	535	AF181859.1	*Aspergillus fumigatus*	98
39	*Fusarium equiseti*	513	510	MH578583	*Fusarium equiseti*	98

Table 3
Fungal load of dried fish products sold in the Zhanjiang market, China.

Fungal isolate	Total fungal load (cfu/g)/number of isolates (n = 10)						
	H.maculatus	P.crocea	L.erythopterus	*T. thynnus*	S.niphonius	E.tetradactylum	T. lepturus
A. flavus	$2.40 \pm 0.32 \times 10^4$	$1.75 \times \pm 0.48 \times 10^3$	$3.20 \pm 0.53 \times 10^3$	$1.37 \pm 0.23 \times 10^3$	$5.23 \pm 1.28 \times 10^3$	$1.10 \pm 0.06 \times 10^3$	$4.45 \pm 2.10 \times 10^3$
A. niger	$1.33 \pm 0.77 \times 10^3$	-[a]	$3.10 \pm 0.38 \times 10^3$	$1.90 \pm 0.25 \times 10^2$	–	$1.25 \pm 0.22 \times 10^2$	$3.31 \pm 0.28 \times 10^2$
A.fumigatus	–	–	–	–	$7.21 \pm 1.76 \times 10^3$	–	–
P.citrinum	–	$4.58 \pm 1.37 \times 10^4$	$1.85 \pm 0.55 \times 10^3$	–	–	–	$1.23 \pm 0.04 \times 10^3$
P.christenseniae	–	$8.11 \pm 3.93 \times 10^3$	–	$3.69 \pm 1.72 \times 10^4$	$2.10 \pm 1.00 \times 10^3$	–	–
P.oxalicum	$3.67 \pm 0.11 \times 10^3$	–	$2.23 \pm 0.08 \times 10^3$	–	–	$2.17 \pm 0.24 \times 10^3$	–
F. equiseti	–	–	$3.19 \pm 0.47 \times 10^2$	$6.32 \pm 2.91 \times 10^3$	$5.53 \pm 1.08 \times 10^2$	–	–
F.oxysporum	–	–	–	$1.09 \pm 0.05 \times 10^2$	–	–	$2.20 \pm 1.05 \times 10^3$
F. chlamydosporum	$2.11 \pm 0.66 \times 10^4$	–	$2.10 \pm 0.74 \times 10^2$	$1.02 \pm 0.45 \times 10^3$	–	$3.11 \pm 1.17 \times 10^2$	–
F. incarnatum	–	$3.76 \pm 1.38 \times 10^3$	–	–	$1.48 \pm 0.23 \times 10^2$	–	–
F. verticillioides	$1.66 \pm 0.28 \times 10^2$	–	–	$3.74 \pm 1.02 \times 10^3$	–	$1.11 \pm 0.16 \times 10^2$	–
F. nelsonii	–	–	–	–	–	$3.02 \pm 1.43 \times 10^3$	–
F.avenaceum	–	–	$1.00 \pm 0.08 \times 10^2$	–	–	$3.22 \pm 0.42 \times 10^3$	–
T. harzianum	$1.23 \pm 0.06 \times 10^3$	–	–	–	$4.36 \pm 1.84 \times 10^3$	–	–
K. ohmeri	$2.10 \pm 0.25 \times 10^2$	–	–	–	–	–	–
C.mucoides	–	–		$1.24 \pm 0.14 \times 10^2$	–	–	–
N. diffluens	–	–	$1.07 \pm 0.04 \times 10^2$	–	–	$1.10 \pm 0.29 \times 10^2$	–
P. curvata	–	–	–	–	$6.11 \pm 2.13 \times 10^2$	–	–
N. dimidiatum	–	–	–	$2.64 \pm 0.49 \times 10^3$	$7.83 \pm 4.93 \times 10^2$	–	–
P. noxious	–	–	–	$1.53 \pm 0.22 \times 10^2$	–	–	–
C. lacerata	–	–	–	$1.10 \pm 0.25 \times 10^2$	–	–	–
N.sphaerica	–	–	$2.53 \pm 0.63 \times 10^2$	–	–	–	–

[a] -:no growth.

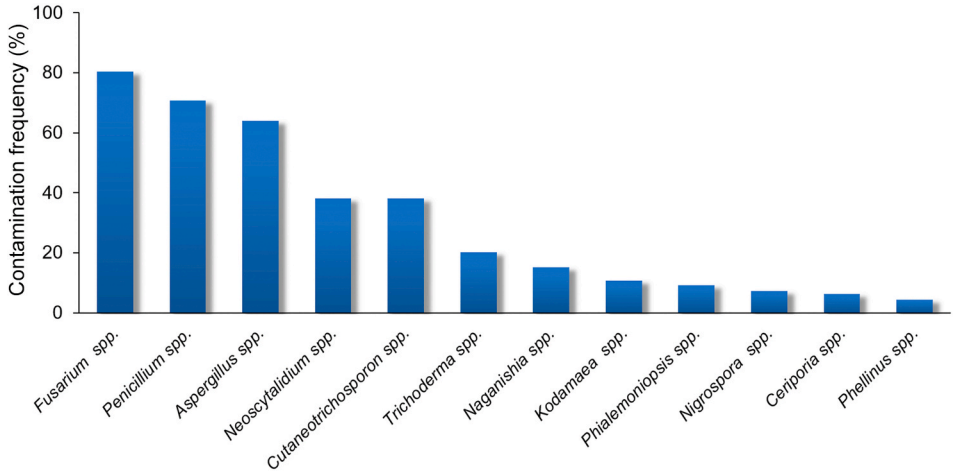

Fig. 4. Contamination frequency (%) of different fungal species in dried fish samples.

10^3–$3.67 \pm 0.11 \times 10^3$ cfu/g respectively were observed. The contamination frequency of *Penicillium* sp. in dried fish was 70.7%, which is higher than the *Aspergillus* sp. (Fig. 4). In many dried fish research, *Penicillium* sp. contamination appears inevitable (Atef et al., 2011; Wogu Iyayi, 2011). *P. citrinum*, *P. oxalicum* and *P. christenseniae*, *P. digitatum* and *P. chrysogenum* have also been reported in smoked dried fish (Job, Agina, & Dapiya, 2016; Oyebamiji & Oyebimpe, 2013). *Penicillium* sp. is saprophytic fungus which mainly grows on rotten fruit, vegetables, meat and various moist organic matters. Hou, Li, and Li (2010) showed that when the humidity increases from 40 to 80%, the toxin-producing ability of *P. citreoviride* is increased. Judet, Bensoussan, Perrier-Cornet, and Dantigny (2008) reported that water activity is important for sporulation and germination of *Penicillium chrysogenum* conidia. Production of conidia enables *Penicillium* sp. to proliferate and spread rapidly on food. During early stages of the drying process, when there is a high moisture content on the fish surface, it provides an ideal condition for rapid *Penicillium* sp. growth.

In this study, seven *Fusarium* sp. were isolated with total fungal counts ranging from $1.00 \pm 0.08 \times 10^2$ to $2.11 \pm 0.66 \times 10^4$ cfu/g in all dried fish samples. *F. chlamydosporum*, *F. verticillioides* and *F. equiseti* were the most frequently detected with the contamination level ranging from $2.10 \pm 0.74 \times 10^2$ to $2.11 \pm 0.66 \times 10^4$, $1.11 \pm 0.16 \times 10^2$–$3.74 \pm 1.02 \times 10^3$ and $3.19 \pm 0.47 \times 10^2$–$6.32 \pm 2.91 \times 10^3$ cfu/g respectively. Although the contamination level of *Fusarium* sp. was lower than the *Aspergillus* sp., the occurrence frequency of *Fusarium* sp. was significantly higher than *Aspergillus* sp.. *Fusarium* contamination in dried fish means the occurrence of trichothecene mycotoxins, which is should not be neglected. The occurrence of *Fusarium* sp. in smoked dried fish has also been reported in other countries (Job, Agina & ;Dapiya, 2016; Fafioye, Efuntoye & ;Osho, 2002) but the contamination levels were not as high as samples from Zhanjiang market in China. That may due to the high humidity (>90%) most of the year, which is ideal for the growth and reproduction of *Fusarium* sp., especially on a high protein medium. Detection of *Fusarium* sp. in dried fish implies a possibility of production of fumonisins, trichothecenes and zearalenone, all of which pose a threat to human health. These mycotoxins are more likely to be produced when dried fish is processed in an unclean environment or poor storage conditions.

In addition to the three dominant fungal genera described above, *Trichoderma* sp. and *Kodamaea* sp. are the others detected in dried fish

(Nyamwaka, Nyamache & Maingi, 2017). In this study, *T. harzianum* were isolated from dried fish with total fungal counts in ranged of $1.23 \pm 0.06 \times 10^3$–$4.36 \pm 1.84 \times 10^3$ cfu/g. *K. ohmeri* was isolated from only one dried fish species with $2.10 \pm 0.25 \times 10^2$ cfu/g. Besides, some uncommon fungi such as *C. mucoides* ($1.24 \pm 0.14 \times 10^2$ cfu/g), *N. diffluens* ($1.07 \pm 0.04 \times 10^2$–$1.10 \pm 0.29 \times 10^2$), *P. curvata* ($6.11 \pm 2.13 \times 10^2$), *N. dimidiatum* ($7.83 \pm 4.93 \times 10^2$–$2.64 \pm 0.49 \times 10^3$), *P. noxious* ($1.53 \pm 0.22 \times 10^2$), *C. lacerata* ($1.10 \pm 0.25 \times 10^2$) and *N. sphaerica* ($2.53 \pm 0.63 \times 10^2$) with relatively low contamination levels were detected in the dried fish samples. These fungi are mostly environmental contaminants. In view of seafood safety, consumption of dried fish contaminated with fungal species such as *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Mucor* sp., and *Rhizopus* sp. can cause health issues as a majority are pathogenic (Paterson & Lima, 2017). There are many factors that influence the dried fish product safety. Firstly, unhygienic processing environment and undesirable storage conditions such as high temperature and humidity, both of which promote fungal reproduction and diffusion. Secondly, air-exposed drying operation lead to contamination of dried fish products by insects and their secreta and excreta. Thirdly, other storage condition such as oxygen concentration and illumination intensity. In addition, during processing, fishermen and workers touch the fish frequently during processing, which allow a transfer of environmental fungi from their hands to the fish surface. Also, consumers inadvertently transfer some fungi to the products when they select them by hand.

3.4. Mycotoxin contamination in dried fish

For optimization of the MS/MS parameters, AFB_1, T-2, OTA and DON were successfully detected. MRM chromatograms of 4 the targeted mycotoxins in the standard solution are shown in Fig. 5. Four contaminating mycotoxins were detected in 12 samples of the 70 tested which amounted to 17.1% detection rate in the dried fish products. The contaminated frequency of AFB_1 and OTA in all samples were higher than T-2 and DON. AFB_1 was detected in 9 samples including 6 dried fish species products (except in the fish *T. thynnus*) with values ranging from 0.03 to 3.52 μg/kg. The AFB_1 contamination rate in dried fish products was 12.8%. T-2 was detected in *L. erythopterus*-S3, *T. thynnus*-S8 and *T. thynnus*-S2 with values of 1.53, 0.86 and 0.21 μg/kg, respectively. OTA was detected in five samples with values ranging from 0.03 to 2.21 μg/kg. DON was found only in *L. erythopterus*-S3 at a concentration of 0.71 μg/kg. The AFB_1, T-2, OTA and DON residues were detected concurrently in *L. erythopterus*-S3 (Table 4). The contamination rate of T-2, OTA and DON in dried fish were 4.28%, 7.14% and 1.42%, respectively.

Aflatoxin is the most ubiquitous and toxic among nearly 400 mycotoxins identified to date. There are four important aflatoxins, AFB_1, AFB_2, AFG_1 and AFG_2 and among these AFB_1 is the most toxic (Liu et al., 2012). The European Commission has set the maximum level for AFB_1 at 2–12 μg/kg to nuts, peanuts, corn and oils seeds (EC, 2010). Chronic exposure to high AFB_1 concentrations can lead to cancer, including hepatocellular carcinoma (Ferreira et al., 2019; Matsuda et al., 2013) and hence a potential health risk to the population consuming dried fish products. Jonsyn & Lahai (1992) identified AFB_1, AFG_1 and AFG_2 in 20 samples of dried smoked fish. Sam et al. (2015) reported that *A. flavus* and *A. niger* isolated from dried fish sold in India by a fuming process produced 0.001–5.492 μg/kg AFB_1 and 0.01–2.96 μg/kg AFG_1, while Fagbohun & Lawal (2015) reported that 2.73–4.03 μg/kg AFB_1 and 2.01–3.53 μg/kg AFG_1 in smoked dried fish sold in Nigeria. Because dried fish has an extended shelf-life, it is a widely consumed and popular food especially in coastal areas because it is freely available. Hence, the regulations need to be more stringent to ensure the quality and safety of dried fish products it is necessary to establish a food safety management system to strictly control drying, storage and selling environments.

The occurrence of T-2, OTA and DON residues in dried fish products were also detected in dried fish sold in the market. Although the contamination level was relatively low, mycotoxins cannot be easily degraded without special treatment. T-2 is the most toxic type A trichothecene mycotoxin, it is an environmental contaminant. A new report shows that reproductive disruption is a key adverse effect of T-2 toxin (Yang et al., 2020). Therefore, detection of T-2 indicated the relatively high health risk of *Fusarium* contamination in dried fish. In this study, we detected the co-occurrence of multiple mycotoxins in dried

Table 4
Contamination levels of mycotoxins in dried fish samples (μg/kg).

Sample	AFB_1	T-2	OTA	DON
H.maculatus-S4	0.05	-[a]	–	–
H.maculatus-S7	–	–	0.24	–
P. crocea-S2	0.09	–	–	–
P. crocea-S5	0.04			
L.erythopterus-S2	0.21[a]	–	0.63	–
L.erythopterus-S3	2.42	1.53	2.21	0.71
T. thynnus-S6	0.32	–	0.03	–
T. thynnus-S8	2.11	0.86	–	–
T. thynnus-S9	3.52	–	–	–
S. niphonius-S2	–	0.21	–	–
S. niphonius-S2	–	–	0.04	–
E.tetradactylum-S1	0.03	–	–	–

[a] Not detected, AFB_1: aflatoxin B_1, T-2: T-2 toxin, OTA: ochratoxin A, DON: deoxynivalenol.

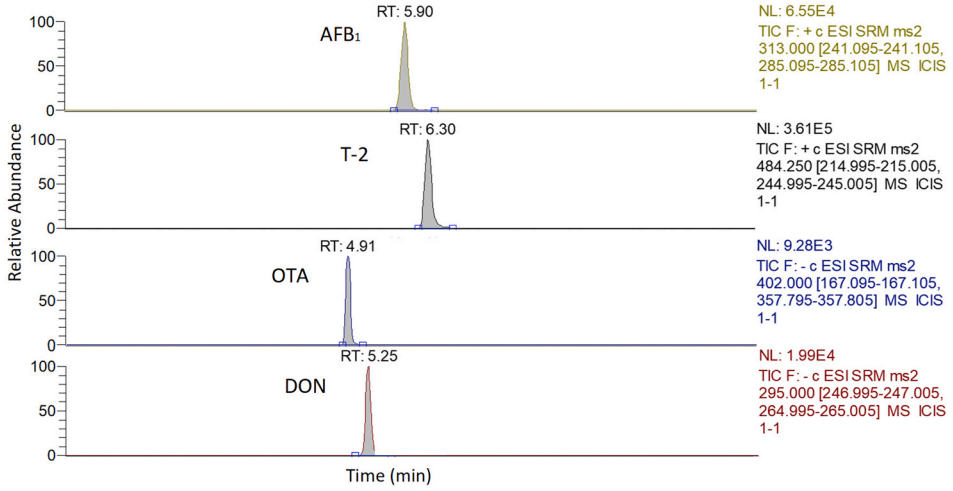

Fig. 5. LC-MS/MS MRM chromatograms of mixed standard solutions of aflatoxin B_1 (AFB_1), T-2 toxin (T-2), ochratoxin A (OTA) and deoxynivalenol (DON).

fish with up to four mycotoxins isolated from some samples. Co-occurrence of mycotoxins is due to at least three different reasons; (i) most fungi are simultaneously able to produce different mycotoxins, (ii) competition among different fungi cause them to secrete fungal toxins to resist invasion by others, and (iii) a self-protective mechanism produced by fungi to survive against adversity (low water activity and high protein) (Alassane-Kpembi et al., 2017). Low level contamination of individual mycotoxins in dried fish is quite common (e.g., AFB_1, OTA and ZEN) and frequently detected (Akinyemi et al., 2011; Gonkowski et al., 2018; Sun et al., 2015). But the co-contamination of mycotoxins in food products is rare. Multi-mycotoxin studies reported foodstuff containing more than one mycotoxin could impact animal and human health at low concentrations (Alassane-Kpembi et al., 2017; Mahdjoubi et al., 2020). Since 1992, the contamination of sun/smoked dried fish products with a single or a combination of mycotoxins has been highlighted (Jonsyn & Lahai, 1992). These findings imply potential health risk to people's health if someone consumes dried fish with multi-fungal contamination. Therefore, it is important for dried fish to be packaged and stored at low temperature, which effectively reduce the probability of air exposure, decrease water activity and inhibit microbial reproduction on dried fish.

4. Conclusion

This is the first investigation of fungal diversity and multi-mycotoxin contamination of the dried fish commodities sold in southern China market. It is also the first report of co-occurrence of four mycotoxins in dried fish sold in the Zhanjiang market. Results showed the presence of toxigenic fungi and mycotoxin residues in the dried fish samples. High contamination frequency of three dominant fungal genera (*Fusarium*, *Aspergillus* and *Penicillium*) pose a health risk due to unhygienically and poorly stored dried fish. The AFB_1, T-2, OTA and DON residues detected in dried fish pose the greatest health concerns. This information will raise awareness and possible lead to guidelines on proper processing methods and the clean storage of dried seafood products, to prevent fungal growth and limit mycotoxin production.

CRediT authorship contribution statement

Yijia Deng: Conceptualization, Formal analysis, Writing - original draft, Visualization. **Yaling Wang:** Funding acquisition, Validation, Writing - review & editing, Supervision. **Qi Deng:** Project administration, Methodology. **Lijun Sun:** Project administration, Formal analysis. **Rundong Wang:** Investigation, Writing - review & editing. **Lin Ye:** Investigation, Software, Formal analysis. **Sen Tao:** Formal analysis. **Jianmeng Liao:** Project administration, Validation. **Ravi Gooneratne:** Writing - review & editing, Supervision.

Declaration of competing interest

The authors would like to declare that there is no conflict of interest in this paper.

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