THE INCIDENCE OF FUNGI AND MYCOTOXINS ASSOCIATED WITH STARCH-BASED FOOD STORED AT DIFFERENT LEVELS OF WATER ACTIVITY

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ABSTRACT

The incidence of fungi and mycotoxin production in ordinary rice grains, glutinous rice grains, riceflour, glutinous riceflour, wheatflour and cornflour stored at different levels of A_w were investigated. In a survey of starch-based food sampled from retail outlets in Malaysia, fungal colonies were mostly detected in wheatflour (100%), followed by riceflour (74%), glutinous rice grains (72%), ordinary rice grains (60%), glutinous riceflour (48%) and cornflour (26%). All positive samples of ordinary rice and glutinous rice grains had total mycoflora count below 10^3 $cfug^{-1}$ sample, while among the riceflour, glutinous riceflour and cornflour samples, 2%, 10% and 4% had more than 10^3 but less than 10^4 $cfug^{-1}$ sample respectively. Sixteen per cent of wheatflour samples had more than 10^3 $cfug^{-1}$ sample and 2% had more than 10^4 $cfug^{-1}$ sample. Aflatoxigenic colonies were mostly detected in wheatflour (20%) samples, followed by ordinary rice grains (4%) and glutinous rice grains (4%) and glutinous riceflour (2%). Samples of riceflour and cornflour were absent from aflatoxigenic colonies. Screening of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2 using reversed-phase HPLC were carried out on 84 samples of ordinary rice grains and 83 samples of wheatflour. Two point four percent (2.4%) of ordinary rice grains were positive for aflatoxin G_1 and 3.6% were positive for aflatoxin G_2. All the positive samples were collected from private homes at concentrations ranging from 3.69 μgkg^{-1} - 77.50 μgkg^{-1}. One point two percent (1.2%) of wheatflour samples were positive for aflatoxin B_1 at a concentration of 25.62 μgkg^{-1}, 4.8% were positive for aflatoxin B_2 at concentrations ranging from 11.25 - 252.50 μgkg^{-1}, 3.6% were positive for aflatoxin G_1 at concentrations ranging from 25.00 - 289.38 μgkg^{-1} and 13.25% were positive for aflatoxin G_2 at concentrations ranging from 16.25 - 436.25 μgkg^{-1}. Similarly, positive wheatflour samples were mostly collected from private homes.

From the water vapour adsorption isotherms, the critical moisture content (% dry basis) i.e. the moisture content to be maintained at 25 °C that will not allow fungal growth was found to be 13.01% for ordinary rice grains, 12.87% for glutinous rice grains, 9.56% for riceflour, 10.61% for glutinous riceflour, 10.72% for wheatflour and 10.52% for cornflour. Correspondingly, in order to maintain

these moisture contents levels, starch-based food must be stored at water activity level of not more than 0.65 equivalent to an equilibrium relative humidity of 65%. However, from this study, at 0.65 A_w, visible appearance of fungi still occurred after 57 days for ordinary rice grains and 73 days for glutinous rice grains. Hence, for ordinary rice and glutinous rice grains, a moisture content lower than 13.01% and 12.87% must be maintained respectively for long-term storage. As water activity of starch-based food increases the development of fungi occurred earlier.

In this study, storage of starch-based food at different levels of water activity at 25 °C for 96 days indicate that the most common genera isolated were *Penicillium* and its teleomorphs (22 species) and *Aspergillus* and its teleomorphs (13 species). The others were zygomycetes (6 species) and *Curvularia lunata*, *Dreschlera* sp., *Moniliella* sp., *Monascus mucoroides* and *Trichoderma* sp. The dominant fungal species occurring on ordinary rice grains were *A. candidus*, *A. flavus*, *A. niger*, *Rhizopus arrhizus* and *R. microsporus*. A significant increase in the incidence from day 0 by *A. candidus* occurred at water activity levels of 0.65, 0.80, 0.85 and 0.98, by *A. flavus* at 0.75 A_w and by *R. arrhizus* at 0.75 and 0.90 A_w. The dominant fungal species occurring on glutinous rice grains were *A. flavus*, *A. niger*, *R. arrhizus* and *R. microsporus*. A significant increase in the incidence from day 0 by *A. flavus* occurred at 0.75 A_w and by *R. microsporus* at all water activities except 0.85. The dominant fungal species occurring on riceflour were *A. flavus*, *A. niger*, *A. terreus*, *P. chrysogenum*, *R. arrhizus* and *R. microsporus*. A significant increase in the incidence from day 0 by *A. flavus* and *R. arrhizus* occurred at water activity levels of 0.65 - 0.90, by *A. niger* at 0.90 A_w, by *A. terreus* and *P. chrysogenum* at all water activity levels and by *R. microsporus* at 0.75 and 0.85 A_w. The dominant fungal species occurring on glutinous riceflour were *A. flavus*, *A. niger*, *P. chrysogenum*, *R. arrhizus* and *R. microsporus*. A significant increase in the incidence from day 0 by *A. flavus* occurred at all water activity levels except at 0.95, by *A. niger* at all water activity levels, by *P. chrysogenum* at 0.80 - 0.98 A_w, by *R. arrhizus* at 0.80 and 0.85 A_w and by *R. microsporus* at 0.90 A_w. The dominant fungal species occurring on wheatflour were *A. candidus*, *A. flavus* and *P. chrysogenum*. A significant increase in the incidence from day 0 by *A. candidus* occurred at all water activity levels, by *A.*

flavus at 0.75 and 0.80 A_w and by *P. chrysogenum* at 0.65, 0.80, 0.85, 0.90 and 0.98 water activity levels. Only *P. chrysogenum* was dominant on cornflour and a significant increase in the incidence from day 0 occurred at all water activity levels.

The dominant fungal species isolated from starch-based food played an important role in the biodeterioration of starch-based food since they have high growth rates ranging from 2.4 $mmday^{-1}$ colony diameter for *A. terreus* to 31.8 $mmday^{-1}$ colony diameter for *R. microsporus*. *Rhizopus arrhizus* was the fastest growing, covering the petri dish in 2 days on starch agar at 25 °C. Furthermore, most of these species were found to exhibit amylolytic activity hence capable of degrading starch.

Mycotoxin extraction and detection, by reversed-phase HPLC using C_{18} column and elution solvent consisting of water and 0.05% TFA in acetonitrile, was evaluated. The extraction method gave good recoveries i.e > 90% for patulin, > 91% for aflatoxin G_1, > 92% for aflatoxin B_1, > 83% for griseofulvin, > 89% for cytochalasin E, > 88% for ochratoxin A and > 90% for sterigmatocystin. The reversed-phase HPLC was able to detect and quantitate seven mycotoxins in 34 min and the retention times obtained was highly reproducible. It was most sensitive for patulin, griseofulvin, aflatoxin G_1, sterigmatocystin, griseofulvin, aflatoxin B_1 and ochratoxin A being able to detect nanogram amounts of the toxin.

Storage of starch-based food at different levels of water activities resulted in the production of various types of mycotoxins at A_w above 0.80 and usually after a period of 26 days and above. Aflatoxin B_1 was the most common mycotoxin present in the starch-based food studied.

Among the starch-based food studied, the highest toxicity was exhibited by glutinous riceflour stored at 0.98 A_w for 54 days, followed by wheatflour stored at 0.90 Aw for 96 days, followed by ordinary rice grains stored at 0.95 A_w for 96 days, followed by riceflour stored at 0.95 A_w for 54 days, followed by glutinous rice grains stored at 0.85 A_w for 96 days and lastly followed by cornflour stored at 0.90 A_w for 54 days.

ABSTRAK

Kehadiran kulat dan pengeluaran mikotoksin pada beras, beras pulut, tepung beras, tepung beras pulut, tepung gandum dan tepung jagung yang disimpan pada paras aktiviti air (A_w) yang berlainan telah dikaji. Di dalam satu tinjauan makanan berasaskan kanji, pensampelan dari kedai-kedai runcit di Malaysia, menunjukkan koloni kulat paling kerap tumbuh pada tepung gandum (100%), di ikuti dengan tepung beras (74%), beras pulut (72%), beras (60%), tepung beras pulut (48%) dan tepung jagung (26%). Kesemua sampel beras dan beras pulut yang positif mempunyai jumlah kiraan mikoflora kurang daripada 10^3 $cfug^{-1}$ sampel, manakala di antara sampel-sampel tepung beras, tepung beras pulut dan tepung jagung, 2%, 10% dan 4% mempunyai lebih daripada 10^3 tetapi kurang daripada 10^4 $cfug^{-1}$ sampel masing-masing. Enam-belas peratus sampel tepung jagung mempunyai lebih daripada 10^3 $cfug^{-1}$ sampel dan 2% mempunyai lebih daripada 10^4 $cfug^{-1}$ sampel. Koloni-koloni kulat aflatoksigenik paling kerap tumbuh pada sampel tepung gandum (20%), diikuti dengan sampel beras (4%) dan beras pulut (4%) dan tepung beras pulut (2%). Koloni kulat aflatoksigenik tidak tumbuh pada sampel-sampel tepung beras dan tepung jagung. Penskrinan aflatoksin B_1, aflatoksin B_2, aflatoksin G_1 dan aflatoksin G_2 menggunakan 'reversed-phase' HPLC telah dilakukan ke atas 84 sampel beras dan 83 sampel tepung gandum. Dua perpuluhan empat peratus (2.4%) sampel beras di dapati positif mengandungi aflatoksin G_1 dan 3.6% adalah positif mengandungi aflatoksin G_2. Kesemua sampel positif dikutip daripada rumah persendirian dalam julat kepekatan 3.69 - 77.50 μgkg^{-1}. Satu perpuluhan dua peratus (1.2%) sampel tepung gandum di dapati positif mengandungi aflatoksin B_1 dalam kepekatan

25.62 μgkg^{-1}, 4.8% adalah positif mengandungi aflatoksin B_1 dalam julat kepekatan 11.25 - 252.50 μgkg^{-1}, 3.6% adalah positif mengandungi aflatoksin G_1 dalam julat kepekatan 25.00 - 289.38 μgkg^{-1} dan 13.25% adalah positif mengandungi aflatoksin G_2 dalam julat kepekatan 16.25 - 436.25 μgkg^{-1}. Sampel-sampel tepung gandum yang positif juga di kutip daripada rumah persendirian.

Daripada isoterma jerapan wap air (water vapour adsorption isotherm), kandungan kelembapan yang kritikal (% kering) i.e. kandungan kelembapan yang mesti dikekalkan pada suhu 25 °C yang tidak membolehkan pertumbuhan kulat pada beras adalah 13.01%, pada beras pulut adalah 12.87%, pada tepung beras adalah 9.56%, pada tepung beras pulut adalah 10.61%, pada tepung gandum adalah 10.72% dan pada tepung jagung adalah 10.52%. Selari dengan ini, untuk mendapatkan kandungan kelembapan di atas, makanan berasaskan kanji mesti disimpan pada aktiviti air tidak melebihi 0.65 setara dengan kelembapan bandingan keseimbangan 65%. Tetapi daripada kajian ini, pada A_w 0.65, kulat mula kelihatan dengan nyata selepas 57 hari untuk beras dan 73 hari untuk beras pulut. Oleh itu, untuk beras dan beras pulut, kandungan kelembapan kurang daripada 13.01% dan 12.87% masing-masing mesti dikekalkan untuk penyimpanan jangka panjang. Perkembangan kulat terjadi dengan lebih cepat apabila penyimpanan pada paras aktiviti air meningkat.

Dalam kajian ini, penyimpanan makanan berasaskan kanji pada paras aktiviti air berlainan pada suhu 25 °C untuk 96 hari menunjukkan genera yang paling kerap dipencil adalah *Penicillium* dan teleomofanya (22 spesies) dan *Aspergillus* dan teleomofanya (13 spesies). Kulat yang lain adalah zigomiset (6 spesies) dan *Curvularia lunata*, *Dreschlera* sp., *Moniliella* sp., *Monascus*

mucoroides dan *Trichoderma* sp. Spesies kulat yang dominan pada beras adalah *A. candidus*, *A. flavus*, *A. niger*, *Rhizopus arrhizus* dan *R. microsporus*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. candidus* berlaku pada aktiviti air 0.65, 0.80, 0.85 dan 0.98, oleh *A. flavus* pada A_w 0.75 dan oleh *R. arrhizus* pada aktiviti air 0.75 dan 0.90. Spesies kulat yang dominan pada beras pulut adalah *A. flavus*, *A. niger*, *R. arrhizus* dan *R. microsporus*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. flavus* berlaku pada A_w 0.75 dan oleh *R. microsporus* pada semua paras aktiviti air melainkan 0.85. Species kulat yang dominan pada tepung beras adalah *A. flavus*, *A. niger*, *A. terreus*, *P. chrysogenum*, *R. arrhizus* dan *R. microsporus*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. flavus* dan *R. arrhizus* berlaku pada paras aktiviti air 0.65 - 0.90, oleh *A. niger* pada A_w 0.90, oleh *A. terreus* dan *P. chrysogenum* pada kesemua paras aktiviti air dan oleh *R. microsporus* pada A_w 0.75 dan 0.85. Spesies kulat yang dominan pada tepung beras pulut adalah *A. flavus*, *A. niger*, *P. chrysogenum*, *R. arrhizus* dan *R. microsporus*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. flavus* berlaku pada kesemua paras aktiviti air melainkan 0.95, *A. niger* pada kesemua paras aktiviti air, oleh *P. chrysogenum* pada A_w 0.80 - 0.98, oleh *R. arrhizus* pada 0.80 dan 0.85 dan oleh *R. microsporus* pada A_w 0.90. Spesies kulat yang dominan pada tepung gandum adalah *A. candidus*, *A. flavus* dan *P. chrysogenum*. Perbezaan peningkatan kehadiran yang nyata daripada hari 0 oleh *A. candidus* berlaku pada kesemua paras aktiviti air, oleh *A. flavus* pada A_w 0.75 dan 0.80 dan oleh *P. chrysogenum* pada paras aktiviti air 0.65, 0.80, 0.85, 0.90 dan 0.98. Hanya *P. chrysogenum* yang dominan pada tepung jagung dan

perbezaan peningkatan kehadiran yang nyata daripada hari 0 berlaku pada semua paras aktiviti air.

Kulat-kulat yang dominan pada makanan berasaskan kanji memainkan peranan yang penting dalam biopereputan oleh kerana ia mempunyai kadar pertumbuhan yang tinggi di antara 2.40 $mmhari^{-1}$ diameter koloni untuk *A. terreus* sehingga 31.83 $mmhari^{-1}$ diameter koloni untuk *R. microsporus*. *Rhizopus arrhizus* mempunyai kadar pertumbuhan paling tinggi dengan memenuhi piring petri dalam masa 2 hari atas agar kanji pada suhu 25 °C. Kebanyakkan spesies ini di dapati mempunyai aktiviti amilolitik iaitu boleh menghurai bahan kanji.

Kaedah pengekstrakan dan pengesanan mikotoksin dengan 'reversed-phase' HPLC menggunakan kolum C_{18} dan pelarut elusi yang terdiri daripada air dan 0.05% TFA dalam asetonitril telah dinilai. Kaedah pengekstrakan yang digunakan memberi penghasilan balik yang tinggi i.e. > 90% untuk patulin, > 91% untuk aflatoksin G_1, > 92% untuk aflatoksin B_1, > 83% untuk griseofulvin, > 89% untuk cytochalasin E, > 88% untuk okratoksin A dan > 90% untuk sterigmatosistin. HPLC 'reversed-phase' boleh mengesan dan kuantitat tujuh mikotoksin dalam masa 34 min dan masa retensi (retention time) yang di dapati adalah boleh ulang. Ianya paling sensitif untuk patulin, griseofulvin, aflatoksin G_1, sterigmatosistin, griseofulvin, aflatoksin B_1 dan okratoksin A iaitu boleh mengesan sehingga nanogram toksin.

Penyimpanan makanan berasaskan kanji pada paras aktiviti air yang berlainan menyebabkan pengeluaran beberapa jenis mikotoksin pada A_w lebih daripada 0.80 pada jangkamasa lebih daripada 26 hari. Aflatoksin B_1 adalah paling kerap hadir dalam makanan berasaskan kanji yang dikaji.

Di antara makanan berasaskan kanji yang dikaji, ketoksikan yang paling tinggi ditunjukkan oleh tepung beras pulut setelah disimpan pada A_w 0.98 selama 54 hari, di ikuti dengan tepung gandum setelah disimpan pada A_w 0.90 selama 96 hari, di ikuti dengan beras setelah disimpan pada A_w 0.95 selama 96 hari, di ikuti dengan tepung beras setelah disimpan pada A_w 0.95 selama 54 hari, di ikuti dengan beras pulut setelah disimpan pada A_w 0.85 selama 96 hari dan akhir sekali oleh tepung jagung setelah disimpan pada A_w 0.90 selama 54 hari.

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LIST OF ABBREVIATIONS

A_w	: water activity
ATA	: Alimentary Toxic Aleukia
BET	: Brunauer-Emmett Teller
cfu	: colony forming units
$cfug^{-1}$	: colony forming units per gram
CCL_4	: carbon tetrachloride
CDA	: Czapek-dox agar
$CHCL_3$	: chloroform
CO_2	: carbon dioxide
°C	: degree Celcius
ERH	: Equilibrium relative humidity
g	: grams
HPLC	: High Performance Liquid Chromatography
hrs	: hours
IARC	: International Agency for Research on Cancer
ICMSF	: International Commission on Microbiological Specification for Foods
KNO_3	: potassium nitrate
LC_{50}	: fifty percent lethal concentration
MARDI	: Malaysian Agricultural Research and Development Institute
MEA	: Malt extract agar
μgg^{-1}	: microgram per gram
μgkg^{-1}	: microgram per kilogram
μgml^{-1}	: microgram per millilitre
µl	: microlitre
µm	: micrometre
mins	: minutes
$mgml^{-1}$	: milligram per millilitre
$mlmin^{-1}$	: millilitre per minute
mm	: millimetre
$mmday^{-1}$	: millimetre per day
$(NH_4)SO_4$	: ammonium sulphate
O_2	: oxygen
PDA	: Potato dextrose agar
S.E.	: standard error
TFA	: Trifluoroacetic acid
U.S.A.	: United States of America
Vps	: vapour pressure of water in food
Vpw	: vapour pressure of pure water
WHO	: World Health Organisation