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PREVALENCE AND CONTROL OF Aspergillus spp. AND AFLATOXINS IN PEANUT SAUCE DURING FOOD PROCESSING

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By

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Chair: Prof. Jinap Selamat, PhD
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Peanut sauce is one of Malaysian cooking dishes which is mass-produced by several companies. Peanut sauce is usually made of peanuts and chili, and these commodities are normally contaminated with *Aspergillus* spp. and aflatoxins (AFs). Unfortunately, the safety of peanut sauce is not always assured as the processing line is not controlled (especially those of small enterprises). Hence, the first objective of this study was to determine the prevalence of *Aspergillus* spp. and AFs along processing steps of peanut sauce manufactured by different companies. Samples were collected from each step of peanut sauce processing at three peanut sauce companies (A, B, and C). Media used to identify *A. flavus* and *A. parasiticus* was *A. flavus* and *A. parasiticus* agar (AFPA), meanwhile AFs were analyzed by using High-performance Liquid Chromatography with a multi λ fluorescence detector (HPLC-FLD). None of the samples from Company A were contaminated with AFs and *Aspergillus* spp. 12.5% of the samples from Company B were contaminated with *Aspergillus* spp. and exceeded the limit. Meanwhile, 87.5% of the samples were contaminated with AFs (0.58 – 32.91 ng/g), with 12.5% of them exceeded the permissible limit. For Company C, 37.5% of the samples were contaminated with *Aspergillus* spp. and exceeded the limit (log 3.44 - 5.05 CFU/g). Meanwhile, all the samples were contaminated with AFs (1.71 – 537.09 ng/g), with 75% of them exceeded the permissible limit. The steps in reducing AFs significantly in the peanut sauce were (i) safety monitoring of raw materials, (ii) sorting of peanut kernels, and (iii) heat treatment of peanut kernels (oil-less frying or frying) and cooking of chili paste or peanut sauce. The second objective of the present study was to control the practices for the reduction of AFs hazards and the effect of interventions in peanut sauce processing. Company C was chosen to fulfill this objective because the level of total AFs in the samples was the highest compared to other companies. Four designs of processing steps were set up and samples were collected according to these designs: (1) control; (2) oil-less frying of chili powder; (3) addition of retort processing; (4) combination of oil-less frying of chili powder and retort processing. Designs 2 and 4 reduced *Aspergillus* spp. significantly in oil-less fried chili powder (from log 3.26 to <3.00 CFU/g), and Design 4 reduced *Aspergillus* spp. in peanut sauce by 15% (from log 3.62 to 3.08 CFU/g). Meanwhile, Design 2 reduced total

AFs by 33% (from 19.98 to 13.48 ng/g), Design 3 reduced total AFs in peanut sauce by 49% (from 363.35 to 184.34 ng/g), while Design 4 significantly reduced total AFs in oil-less fried chili and peanut sauce by 41% (from 30.00 to 17.60 ng/g) and 57% (from 384.74 to 164.62 ng/g), respectively. This study proved that oil-less frying of chili powder, retort processing, and a combination these processes significantly reduce AFs levels. Design 4 (combination of oil-less frying of chili powder and retort processing) yielded the highest reduction of total AFs and therefore recommended to be employed by the Company C.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

KELAZIMAN DAN KAWALAN *Aspergillus* spp. DAN AFLATOKSIN – AFLATOKSIN DALAM KUAH KACANG SEMASA PEMPROSESAN MAKANAN

Oleh

FARAWAHIDA BINTI ABDUL HALIM

September 2017

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Kuah kacang adalah salah satu makanan rakyat Malaysia yang telah dihasilkan besar-besaran oleh beberapa syarikat. Kuah kacang selalunya diperbuat daripada kacang dan cili, dan komoditi-komoditi ini seringkali dikontaminasi oleh *Aspergillus* spp. dan aflatoksin-aflatoksin (AFs). Malangnya, keselamatan kuah kacang selalunya tidak dijamin kerana jaluran pemprosesan tidak dikawal (terutamanya perusahaan kecil). Oleh itu, objektif pertama kajian ini ialah untuk mengenalpasti kelaziman *Aspergillus* spp. dan AFs sepanjang langkah pemprosesan kuah kacang yang dihasilkan daripada syarikat-syarikat yang berlainan. Sampel-sampel telah diambil daripada setiap langkah pemprosesan kuah kacang daripada tiga syarikat kuah kacang (A, B dan C). Media yang telah digunakan untuk mengenal pasti *A. flavus* dan *A. parasiticus* ialah *A. flavus* and *A. parasiticus* agar (AFPA), manakala AFs telah dianalisa dengan menggunakan Kromatografi Cecair Berprestasi Tinggi bersama sebuah pengesan fluorescent pelbagai λ (HPLC-FLD). Tiada sampel-sampel daripada Syarikat A yang dikontaminasi dengan AFs dan *Aspergillus* spp. 12.5% sampel-sampel daripada Syarikat B telah dikontaminasi dengan *Aspergillus* spp. dan telah melebihi had. Sementara itu, 87.5% daripada sampel-sampel telah dikontaminasi dengan AFs (0.58 - 32.91 ng/g), dengan 12.5% daripadanya telah melebihi had yang dibenarkan. Bagi Syarikat C, 37.5% daripada sampel-sampel telah dikontaminasi dengan *Aspergillus* spp. dan telah melebihi had (log 3.44 – 5.05 CFU/g). Sementara itu, semua sampel telah dikontaminasi dengan AFs (1.71 – 537.09 ng/g), dengan 75% daripadanya telah melebihi had yang dibenarkan. Langkah-langkah bagi mengurangkan AFs yang signifikan dalam kuah kacang ialah (i) pengawalan keselamatan bahan-bahan mentah, (ii) pengasingan kacang tanah, dan (iii) rawatan haba kacang tanah [penggorengan atau penggorengan tanpa minyak (*sangai*)] dan memasak pes cili atau kuah kacang. Objektif kedua kajian semasa ialah untuk mengawal praktis-praktis bagi mengurangkan bahaya-bahaya AFs dan kesan intervensi-intervensi dalam pemprosesan kuah kacang. Syarikat C telah dipilih bagi memenuhi objektif ini kerana tahap jumlah AFs di dalam sampel-sampel adalah yang tertinggi berbanding syarikat-syarikat lain. Empat reka bentuk langkah pemprosesan telah direka dan sampel-sampel telah diambil berdasarkan reka bentuk ini: (1)

kawalan; (2) menggoreng serbuk cili tanpa minyak (*sangai*), (3) penambahan pemprosesan retort; (4) kombinasi kesan menggoreng serbuk cili tanpa minyak (*sangai*) dan pemprosesan retort. Reka Bentuk 2 dan 4 telah mengurangkan *Aspergillus* spp. secara ketara dalam cili yang telah digoreng tanpa minyak (*sangai*) (daripada log 3.26 kepada <3.00 CFU/g), dan Reka Bentuk 4 telah mengurangkan *Aspergillus* spp. dalam kuah kacang sebanyak 15% (daripada log 3.62 kepada 3.08 CFU/g). Sementara itu, Reka Bentuk 2 telah mengurangkan jumlah AFs sebanyak 33% (daripada 19.98 kepada 13.48 ng/g), Reka Bentuk 3 telah mengurangkan jumlah AFs dalam kuah kacang sebanyak 49% (daripada 363.35 kepada 184.34 ng/g), manakala Reka Bentuk 4 secara ketara telah mengurangkan jumlah AFs dalam serbuk cili yang telah digoreng tanpa minyak (*sangai*) dan kuah kacang masing-masing sebanyak 41% (daripada 30.00 kepada 17.60 ng/g) dan 57% (daripada 384.74 kepada 164.62 ng/g). Kajian ini telah membuktikan bahawa menggoreng serbuk cili tanpa minyak (*sangai*), pemprosesan retort, dan kombinasi proses-proses ini secara ketara telah mengurangkan tahap AFs. Reka Bentuk 4 [kombinasi menggoreng serbuk cili tanpa minyak (*sangai*) dan pemprosesan retort] telah menghasilkan pengurangan jumlah AFs yang paling tinggi dan dicadangkan supaya diaplikasikan oleh Syarikat C.

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I certify that a Thesis Examination Committee has met on 25th of September 2017 to conduct the final examination of Farawahida Binti Abdul Halim on her thesis entitled "Prevalence and Control of *Aspergillus* spp. and Aflatoxins in Peanut Sauce during Food Processing" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.A. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Signature: _____
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

~	Approximately
°	Degree
°C	Degree Celsius
Ø	Diameter
>	Greater than
<	Less than
µL	Microliter
µm	Micrometer
×	Multiplication
%	Percentage
±	Plus-minus
-	to
ACN	Acetonitrile
AFB_1	Aflatoxin B_1
AFB_1-epoxide	AFB_1-8,9-epoxide
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFPA	*A. flavus* and *A. parasiticus* agar
AFs	Aflatoxins
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemist
BCE	Before Common Era
BRC	British Retail Consortium
CAC	Codex Alimentarius Commission
CCPs	Critical Control Points
CFU/g	Colony-forming unit per gram
CLs	Critical Limits
cm	Centimeter
COA	Certificate of Analysis
Codex Alimentarius	Codex Alimentarius International Food Standards
CYP	Cytochrome P450 enzymes
DAD	Diode array detector
DNA	Deoxyribonucleic acid
drops/sec	Drops per second
EC	European Commission
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FLD	Fluorescence detector
FSMS	Food Safety Management Systems
g	Gram
g/day	Gram per day
GASGA	Group for Assistance on Systems relating to Grain After-harvest
GC	Gas chromatography
GHP	Good Hygiene Practices

GMP	Good Manufacturing Practices
h	Hour
H_2O	Water
HACCP	Hazard Analysis and Critical Control Point
HPLC	High-performance Liquid Chromatography
HPLC-FLD	High-performance Liquid Chromatography with a multi λ fluorescence detector
IAC	Immunoaffinity chromatography
IARC	International Agency for Research on Cancer
ISO	International Organization for Standardization
kb	Kilobase
kg	Kilogram
km	Kilometer
LOD	Limit of detection
LOQ	Limit of quantification
MA	Massachusetts
MeOH	Methanol
MeSTI	*Makanan Selamat Tanggungjawab Industri*
metric tons/year	Metric tons per year
mil	Million
min	minute
mL	Milliliter
mL/min	Milliliter per min
mm	Millimeter
MOH	The Malaysian Ministry of Health
mRNA	Messenger RNA
MS	Malaysian Standard
MSG	Monosodium glutamate
MYR	Ringgit Malaysia
n	Number of samples
N	Not stated
NaCl	Sodium chloride
NA	Not applicable
ND	Not detected
ng/mL	Nanogram per milliliter
ng/g	Nanogram per gram
ng/kg	Nanogram per kilogram
ng/kg bw/day	Nanogram per kilogram body weight per day
nm	Nanometer
NY	New York
PA	Pennsylvania
PHRED	Photochemical reactor for enhanced detection
PRPs	Prerequisite programs
QA	Quality Assurance
QC	Quality Control
R^2	Coefficient of regression
RNA	Ribonucleic acid
RTE	Ready-to-eat
Standards Malaysia	Department of Standards Malaysia
SK1M	*Skim Keselamatan Makanan 1Malaysia*

SMEs	Small and medium enterprises
SOP	Standard operating procedures
SD	Standard deviation
TLC	Thin-layer chromatography
UK	United Kingdom
U.S. FDA	United States of Food and Drug Administration
USA	The United States of America
UV	Ultraviolet
v/v	Volume per volume
v/v/v	Volume per volume per volume

CHAPTER 1

INTRODUCTION

1.1 Research Background

Aflatoxins (AFs) are toxic, mutagenic, and carcinogenic compounds that might contaminate a wide variety of foods and feedstuffs (Chen et al., 2013). Nearly 20 different types of AFs have been identified, but the four important AFs associated with foods are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) (Bhat et al., 2010; Pittet, 1998). Peanuts (*Arachis hypogaea* L.) are considered to be at high risk of contamination with mycotoxins, especially with AFs because they are susceptible to the attack of *Aspergillus* spp. while peanut pods are drying in the field after uprooting (Zorzete et al., 2011; Horn & Dorner, 1999). According to the International Agency for Research on Cancer (IARC; 1993), AFB_1 is the most toxic form and has been classified as a group 1 carcinogen; while AFB_2, AFG_1, and AFG_2 are categorized as group 2B (possible carcinogens to humans).

Ingestion of peanuts and peanut products which are contaminated with AFs, can increase human exposure to AFs (Iqbal et al., 2013). In the literature, there are many studies reported on the occurrence of AFs in various types of nuts especially groundnuts and peanut products. Many researchers in Malaysia reported high occurrence of AFs in peanut kernels in Malaysia (Afsah-Hejri et al., 2013a; Leong et al., 2010; Arzandeh et al., 2010; Sulaiman et al., 2007). The study by Afsah-Hejri et al. (2013a) found that raw peanut kernels in Malaysia were contaminated with 977.66 ± 12.19 ng/g and 231.09 ± 9.98 ng/g of AFB_1 and AFB_2, respectively. Leong et al. (2010) reported that raw groundnut shelled can be contaminated with 711 ng/g total AFs, while a study from Sulaiman et al. (2010) showed that raw shelled peanuts were contaminated with 762 ng/g total AFs. These contaminations far exceeded the Malaysian regulation as Malaysia only permits 15 ng/g of total AFs in peanut and peanut products (Food Act, 1983).

Peanut sauce is one of the most popular peanut products available in South East Asia. The peanut sauce is also known as satay sauce, *bumbu kacang, sambal kacang* and *sambal pecel*. Peanut sauce is concentrated gravy, brownish in color, and usually consumed with sate (traditional skewered grilled meat; Jinap et al., 2013), *nasi impit* (compact rice*)*, *pecal* (vegetable dish) and others. The ingredients of peanut sauce are peanut kernels, chili, garlic, onion, salt, sugar, tamarind juice and water. The main ingredients of peanut sauce, which are peanut kernels and chili, are favorable for *Aspergillus* spp. growth and AFs contamination. Studies from Jalili & Jinap (2012) and Khayoon et al. (2012) showed that chili from Malaysia could be contaminated with 79.7 ng/g and 44.2 ng/g of total AFs, respectively. These contaminations exceeded the limit set by the regulation, which is 5 ng/g (Food Act, 1983). Due to the high prevalence of AFs in raw peanuts and chili, there is a high possibility that peanut product such as peanut sauce is contaminated with *Aspergillus* spp. and AFs.

Peanut sauce is normally produced at home (home-made), restaurants or street vendors. Recently, several companies start producing and commercialize this product in the form of ready-to-eat (RTE) or pre-mix peanut sauce, depending on the target consumers. According to the information from the Malaysian Ministry of Health (MOH), there are approximately 10 peanut sauce manufacturers available in Malaysia (MOH, personal communication, June 9, 2015). 90% of the manufacturers involved are small and medium enterprises (SMEs) companies while others are medium companies. Due to the differentiation of company profile in term of size, number of workers, equipment used, final products and certification obtained, the process of peanut sauce between the companies varies. However, the general steps involved in peanut sauce processing are raw materials receiving, pre-cleaning, sorting, frying, grinding, cooking, packaging and delivery.

Differentiation of companies profile affected the processing steps of peanut sauce. The owner of the companies who possess the knowledge about AFs and food safety will be more alert about AFs. The companies with Food Safety Management Systems (FSMS) may have financial resources to invest their funds for better instruments, facilities and training. Therefore, the peanut sauce processing in the companies are properly controlled from the receiving of raw materials and this produces end products with a lower AFs level. In contrast, small companies are expected to have a lack of financial resources to improve the instruments, facilities, manpower, workers' knowledge and FSMS. Thus, the process could continue using the manual way for batch processing steps. Manual processing would result in high possibilities of cross-contamination of *Aspergillus* spp. and AFs along the steps in peanut sauce processing.

1.2 Problem Statements

Although the peanut sauce is one of the heritage peanut products widely consumed in Malaysia, there is limited information reported in the literature on the prevalence of *Aspergillus* spp. and AFs in peanut sauce from Malaysia. Most of the studies reported were from Indonesia. Razzazi-Fazeli et al. (2004) reported peanut sauce in Indonesia were contaminated with high level of AFs in the range of 8-207 ng/g and 7-613 ng/g of AFB_1 and total AFs, respectively. Studies from Dharmaputra et al. (2013) also found that peanut sauce was contaminated with total AFs in the range of 0-198.6 ng/g. Due to the limited information available, there is no study to control the practices for the reduction of AFs hazards during processing in peanut sauce companies.

Physical, chemical and biological methods could be introduced in the peanut sauce companies in order to reduce, detoxify or eliminate the AFs effects (Jardon-Xicotencatl et al., 2015; Doyle et al., 1982). There were several rules to be followed for the detoxification method, which were: 1) inactivate, destroy, or reduce the toxin, 2) not produce or leave toxic residues in the food, 3) retain the nutritive value of the food, 4) not alter the acceptability or the technological properties of the product, and, if possible, 5) destroy fungal spores (Park, 2002). Physical methods are convenient to

be adopted to be applied in the peanut sauce companies since these methods are simple, safe and practical to the used in the foods.

1.3 Objectives

There were two objectives in this study. The first objective of this study was to determine the prevalence of *Aspergillus* spp. and AFs along processing steps of peanut sauce manufactured by different companies. The results from the objective then were carried out for the second objective, which was to control the practices for the reduction of AFs hazards and the effect of interventions in peanut sauce processing.

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