

UNIVERSITI PUTRA MALAYSIA

AFLATOXIN BIOMARKERS IN HUMAN BIOLOGICAL SAMPLES AND THEIR POTENTIAL REDUCTION BY PROBIOTIC Lactobacillus casei SHIROTA STRAIN

MOHD REDZWAN BIN SABRAN

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DOCTOR OF PHILOSOPHY UNIVERSITI PUTRA MALAYSIA

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

September 2014

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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By

MOHD REDZWAN BIN SABRAN

September 2014

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Faculty : Medicine and Health Sciences

This thesis comprised of five main research projects, studied the presence of aflatoxin biomarkers in human biological samples and the use of probiotic *Lactobacillus casei* Shirota strain (LcS) as a potential aflatoxin adsorbent. The first research project involved a questionnaire survey among 160 subjects to assess their knowledge on fungal and aflatoxin contamination in the diets. More than half of subjects (n=84, 52.5%) participated in the screening stage had low level of knowledge. There were several significant findings between socio-demographic characteristics and subjects' knowledge on fungal and aflatoxin contamination in the diets. In particular, being female and single and with personal income below RM1500 accounted for a significant 10.6% of the variability in the subjects' overall scores of knowledge on fungal and aflatoxin contamination in the diets [R^2 =0.106, adjusted R^2 =0.089, *F* (2, 156)=6.154, p=0.001] and the personal income was found to be the sole determinant of subjects' overall knowledge (β =-0.288, p=0.000).

As for the second research project, morning urine samples were collected from the subjects for the measurement of Aflatoxin M_1 (AFM₁) using enzyme linked immunosorbent assay (ELISA) as well as to determine its association with the food consumption. Ninety-eight urine samples (n=98) were positive with AFM₁. Only four from 37 food items in the food frequency questionnaire (FFQ) namely ready-to-eat cereals (r=0.222, p=0.036), soybean milk (r=0.266, p=0.011), *kuih kacang* (r=0.222, p=0.035) and peanut butter (r=0.211, p=0.045) showed moderate and positive association with the levels of urinary AFM₁. A significant association (ϕ =0.286) was found between the levels of urinary AFM₁ and the consumption of milk and dairy products as subjects with intake of milk and dairy products greater than 67.78 g/day had significantly and higher urinary AFM₁ levels. The estimated dietary Aflatoxin B₁ (AFB₁) exposure was 11.7 ng/day/ kg body weight, contributing to 0.29 cancer cases in 100, 000 populations where 6.1% of liver cancer could be attributable by aflatoxin exposure.

The third research project pertained to the use of ultra high performance liquid chromatography (UHPLC) for the measurement of urinary AFM_1 . The UHPLC method was optimized and used to analyse urinary AFM_1 among seventy-one subjects (n=71) recruited from 160 subjects that participated in the screening stage. Thirteen subjects (n=13) had detectable urinary AFM_1 ranging from 2.4 to 100.34 pg/ml.

As for the fourth research project, the study was conducted to determine the effectiveness of 4 weeks cross-over intervention study with fermented milk containing LcS in reducing the levels of aflatoxin biomarkers in human blood and urine samples. Seventy-one subjects (n=71) were divided into two groups namely Blue and Yellow group. Overall, the intervention did not significantly reduce the levels of serum AFB₁-lysine adduct and urinary AFM₁ as well as the liver and kidney biomarkers. Nonetheless, the potential of LcS as an aflatoxin adsorbent to a certain extent was observed in some subjects especially in the Blue group. Within 2 weeks of intervention, the levels of serum AFB₁-lysine adduct reduced significantly from 6.24 ± 3.42 pg/mg albumin (ALB) to 5.14 ± 2.15 pg/mg ALB, with 17.63% of reduction. Although not significant (p=0.332), the levels of AFB₁-lysine at the end of intervention (4th week) was lower compared to the baseline levels. As for the urinary AFM₁ levels, a decreasing trend was observed over the 4 weeks of intervention.

The fifth project was conducted to determine the effect of LcS on the bioaccessibility of AFB_1 through an *in vitro* simulation of human digestion under fed condition. Peanut samples were artificially contaminated by spiking with two contamination levels of AFB_1 namely 4.53 and 8.56 ng/g. The contaminated peanut samples were applied to the simulation model together with three treatments namely activated carbon, cultured LcS and probiotic drink containing LcS. The average AFB_1 bioaccessibility of 83.92% from both spiked peanut samples with 4.53 ng/g and 8.56 ng/g indicated that AFB_1 was released completely from the food matrix (i.e peanut samples). The addition activated carbon reduced greatly AFB_1 bioaccessibility. By comparison, the addition of LcS (cultured LcS and probiotic drink containing LcS) did not produce a big reduction of AFB_1 bioaccessibility as seen with the application of activated carbon. Nonetheless, the treatment to a certain extent decreased AFB_1 bioaccessibility about 20% especially in the peanut samples spiked with 8.56 ng/g AFB_1 .

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENANDA BIO AFLATOKSIN DI DALAM SAMPEL BIOLOGI MANUSIA DAN POTENSI PENGURANGANNYA DENGAN PROBIOTIK Lactobacillus casei STRAIN SHIROTA

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Tesis ini merangkumi lima projek penyelidikan utama, mengkaji kehadiran penanda bio aflatoksin di dalam sampel biologi manusia dan penggunaan probiotik Lactobacillus casei strain Shirota (LcS) sebagai penyerap aflatoksin yang berpotensi. Projek penyelidikan pertama melibatkan satu kajian soal selidik di kalangan 160 subjek untuk menilai pengetahuan mereka terhadap pencemaran kulat dan aflatoksin di dalam diet. Lebih separuh daripada subjek (n=84, 52.5%) yang telah mengambil bahagian di dalam peringkat saringan mempunyai tahap pengetahuan yang rendah. Terdapat beberapa penemuan yang signifikan di antara ciri-ciri sosio-demografik dengan pengetahuan subjek mengenai pencemaran kulat dan aflatoksin dalam diet. Khususnya, subjek perempuan dan bujang yang mempunyai pendapatan kewangan peribadi di bawah RM1500 menyumbang dengan signifikan 10.6% daripada kebolehubahan di dalam skor keseluruhan pengetahuan subjek mengenai pencemaran kulat dan aflatoksin di dalam diet $[R_2=0.106, R_2 \text{ diselaraskan}=0.089, F(2, 156)= 6.154, p=0.001]$ dan pendapatan kewangan peribadi merupakan penentu tunggal bagi pengetahuan keseluruhan subjek (β =-0,288, p=0.000).

Bagi projek penyelidikan kedua, sampel urin pagi telah dikumpul daripada subjek untuk pengukuran Aflatoksin M_1 (AFM₁) menggunakan penetapan kadar imunosorben taut-enzim (ELISA) serta untuk menentukan perkaitannya dengan pengambilan makanan. Sembilan puluh lapan sampel urin (n=98) adalah positif dengan AFM₁. Hanya empat daripada 37 barangan makanan di dalam soal selidik kekerapan makanan (FFQ) iaitu bijirin sedia ada (r=0.222, p=0.036), susu kacang soya (r=0.266, p=0.011), kuih kacang (r=0.222, p=0.035) dan mentega kacang (r=0.211, p=0.045) menunjukkan perkaitan yang sederhana dan positif dengan tahap AFM₁ di dalam urin. Satu hubungan yang signifikan (ϕ =0.286) didapati antara tahap AFM₁ di dalam urin dan pengambilan susu dan produk tenusu di mana subjek dengan pengambilan susu dan produk tenusu lebih daripada 67.78 g/hari mempunyai tahap AFM₁ di dalam urin yang lebih tinggi dan signifikan. Anggaran pendedahan Aflatoksin B₁ (AFB₁) melalui makanan adalah 11.7 ng/hari/kg berat badan, menyumbang kepada 0.29 barah di dalam 100, 000 penduduk di mana 6.1% daripada barah hati boleh berpunca daripada pendedahan aflatoksin.

Projek penyelidikan ketiga adalah mengenai penggunaan kromatografi cecair prestasi ultra tinggi (UHPLC) bagi pengukuran AFM_1 di dalam urin. Kaedah UHPLC ini telah dioptimumkan dan digunakan untuk menganalisis AFM_1 di dalam urin bagi tujuh puluh satu subjek (n=71) yang dipilih daripada 160 subjek yang terlibat di dalam peringkat saringan. Tiga belas subjek (n=13) mempunyai tahap AFM_1 di dalam urin yang boleh dikesan, berada di antara 2.4 sehingga 100.34 pg/mL.

Bagi projek penyelidikan yang keempat, kajian dijalankan untuk menentukan keberkesanan intervensi kajian silang selama 4 minggu dengan susu fermentasi yang mengandungi LcS untuk mengurangkan tahap penanda bio aflatoksin di dalam sampel darah dan urin manusia. Tujuh puluh satu subjek (n=71) telah dibahagikan kepada dua kumpulan iaitu kumpulan Biru dan Kuning. Secara keseluruhan, intervensi tersebut tidak dapat mengurangkan secara signifikan tahap serum aduk AFB₁-lisin dan AFM₁ di dalam urin serta penanda bio hati dan buah pinggang. Walaubagaimanapun, potensi LcS sebagai penyerap aflatoksin sedikit sebanyak dapat diperhatikan di dalam beberapa subjek terutamanya di dalam kumpulan Biru. Dalam tempoh 2 minggu intervensi, tahap serum aduk AFB₁-lisin berkurang dengan signifikan daripada 6.24 ± 3.42 pg/mg albumin (ALB) kepada 5.14 ± 2.15 pg/mg ALB, dengan pengurangan sebanyak 17.63%. Walaupun tidak signifikan (p=0.332), tahap aduk AFB₁-lisin di akhir intervensi (minggu ke-4) adalah lebih rendah berbanding dengan tahapnya di permulaan. Bagi tahap AFM₁ di dalam urin, satu trend penurunan dapat diperhatikan sepanjang tempoh 4 minggu intervensi.

Projek kelima dijalankan untuk menentukan kesan LcS terhadap kebolehcapaian bio AFB1 melalui simulasi in vitro pencernaan manusia di bawah keadaan diberi makan. Sampel kacang telah dicemarkan dengan mencampurkan AFB₁ pada dua tahap pencemaran iaitu sebanyak 4.53 dan 8.56 ng/g. Kacang yang dicemarai itu telah melalui model simulasi bersama tiga rawatan iatu dengan karbon yang diaktifkan, kultur LcS dan minuman probiotik yang mengandungi LcS. Purata kebolehcapaian bio AFB₁ adalah 83.92%, daripada kedua-dua sampel kacang yang dicampurkan dengan 4.53 ng/g dan 8.56 ng/g menunjukkan bahawa AFB₁ dibebaskan sepenuhnya dari matriks makanan (iaitu sampel kacang). Penambahan karbon yang diaktifkan mengurangkan kebolehcapaian bio AFB1 dengan ketara. Sebagai perbandingan, penambahan LcS (kultur LcS dan minuman probiotik yang mengandungi LcS) tidak menghasilkan pengurangan yang besar bagi kebolehcapaian bio AFB₁ seperti yang dilihat dengan rawatan oleh karbon yang diaktifkan. Walaubagaimanapun, rawatan tersebut untuk satu tahap tertentu dapat menurunkan kebolehcapaian bio AFB1 lebih kurang 20% terutamanya di dalam sampel kacang yang dicampurkan dengan 8.56 ng/g AFB₁.

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LIST OF ABBREVIATIONS

AAD	Antibiotic-associated diarrhoea
AFB_1	Aflatoxin B_1
AFB ₁ -NAC	Aflatoxin B_1 mercapturic acid
AFB_2	Aflatoxin B_2
AFG ₁	Aflatoxin G ₁
AFG ₂	Aflatoxin G ₂
AFL	Aflatoxicol
AFM_1	Aflatoxin M_1
AFM _{2a}	AFM ₁ hemiacetal derivative
AFP ₁	Aflatoxin P ₁
AFQ ₁	Aflatoxin Q_1
AFs	Aflatoxins
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ANOVA	Analysis of variance
AOAC	Association of analytical communities
AST	Aspartate Aminotransferase
ATA	Alimentary toxic aleukia
AUC	Area under curve
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
BSA	Bovine serum albumin
BUN	Blood Urea Nitrogen
CFU	Colony forming unit
CI	Confidence interval
CKD	Chronic kidney disease
CV	Coefficient of variance
DIO	Diet-induced obesity
DMA	Data Medical Associates
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
ELISA	Enzyme-linked immunosorbent assay
FFQ	Food frequency questionnaire
FUMB ₁	Fumonisim B_1
GGT	Gamma-glutamyl transpeptidase
GSH	Glutathione
GST	Glutathione-s-transferase
HBsAg	Non hepatitis B-positive or Hepatitis B-negative
HbsAg ⁺	Hepatitis B-positive
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HPLC	High performance liquid chromatography
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer
IBS	Irritable bowel syndrome
IFN-γ	Interferon-y
IL-10	Interleukin 10
IL-12	Interleukin 12

IL-2	Interleukin 2
IL-6	Interleukin 6
ITT	Insulin tolerance test
ITT	Intention to treat
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
KMO	Kaiser-Meyer-Olkin
LAB	Lactic acid bacteria
LBP	Lipopolysaccharide-binding protein
LcS	Lactobacillus casei Shirota strain
LGG	Lactobacillus rhanmosus strain GG
LOD	Limit of detection
LOQ	Limit of quantification
М	Molar
mEH	Microsomal epoxide hydrolase
MeOH	Methanol
MOH	Ministry of Health
MRA	Multiple regression analysis
MRS	de Man, Rogosa and Sharpe
NCBI	National Centre for Biotechnology Information
ND	Not determined
NHS	Normal human serum
NIV	Nivalenol
NK	Natural killer
NMRR	National Medical Research Registry
NS	Not stated
OA	Ovalbumin
OCH ₃	Oxy-methyl
-OH	Hydroxyl
OR	Odds ratio
OTA	Ochratoxin A
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI	Phase 1
PII	Phase 2
PS	Polysaccharide
QC	Quality control
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RSM	Response surface methodology
SAR	Seasonal allergic rhinitis
SCFA	Short chain fatty acid
SCT	Socio Cognitive Theory
SD	Standard deviation
SD_{pooled}	Pooled standard deviation
SDS	Sodium dedocyl sulphate
SD_w	Within subject variance
SPE	Solid phase extraction
TSAW	Trichinella spiralis adult worms
UHPLC	Ultra high performance liquid chromatography

UK	United Kingdom
UPM	Universiti Putra Malaysia
URTI	Upper respiratory-tract infection
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

1.1. Background of the study

All living lives require sustainable food supply for survival in the upcoming and challenging world where the food resources could be limited and contaminated. Moreover, the emergence of new diseases for the past decades and the discovery of a wide range of diseases associated with the diets show how vulnerable humans are (Keesing *et al.*, 2010; Giraud *et al.*, 2010; Newell *et al.*, 2010). Even so, these phenomena can be avoided if humans can maintain and achieve maximum health benefits through the healthy and nutritious diets and safe foods. On the other hand, it is also believed that not everyone is particular on the importance of healthy food eating and food safety (Steeves *et al.*, 2012; Newell *et al.*, 2010). In one of many instances, people's awareness on food safety is still below par as evident by many cases of food-borne illnesses and poisonings that happened around the globe (Newell *et al.*, 2010). It is indeed a top priority issue and certainly a public concern as the incidences are not only detrimental to the human well-being, but economically affect many nations especially among the developing countries such as in Asia and Africa (Negedu *et al.*, 2011).

Malaysia, is one the countries that is facing the same dilemma. It is apparent by the publication of reports and articles on the occurrence of contaminants and toxicants in the diets and their impacts to humans and animals. In fact, one of the evidences that can be seen in Malaysia is the aflatoxicosis case that occurred in Perak in 1988, which has led to thirteen deaths of children (Lye *et al.*, 1995). The occurrence of aflatoxin, one of the mycotoxin produced by fungi is one of many toxicants that are commonly found in the human food resources and the significant impact of this food-borne illness should not be taken for granted. As in Malaysia, there are studies reported the contamination of aflatoxin in the foodstuffs (Leong *et al.*, 2010; Arzandeh *et al.*, 2010; Sulaiman *et al.*, 2007). For example, a recent study by Samsudin and Abdullah (2013) measured aflatoxin contamination level ranged from 0.61 to 77.3 μ g/kg in red rice and 35 of 50 samples analysed had levels higher than the Malaysian and European standard of 5 and 4 μ g/kg respectively.

The example is one of many mycotoxicosis cases that happened around the world due to fungal infections. In the tenth century, the infection of fungi in the diets caused the outbreak of disease known as St. Anthony's or Holy Fire in many European countries due to the contamination of rye by ergot alkaloid, produced by *Claviceps purpurea* (Paterson & Lima, 2010a). Since then, many cases have been reported and the discovery of "Turkey X" disease caused by aflatoxin, in 1950s and early 1960s had opened new prospectus on the scientific research on the aetiology of mycotoxicosis and preventive strategies in foods, animals and humans (Kensler

et al., 2011). Kensler et al. (2011) described the epidemic disease as the major cause of death of numerous poultry animals including ducklings and chicks due to the consumption of diet containing contaminated peanuts and exposed to Aspergillus flavus, a pathogenic fungus. It was found that extracts from the culture of the fungus isolated from the meal were found to have the capability to induce the "Turkey X" syndromes (Kensler et al., 2011). Due to this, most of the reported cases on mycotoxicosis focused on the specific species of fungi and four major species of fungi have been discovered belonging to the species of Aspergillus, Fusarium, Penicillium and Claviceps that produced some major mycotoxins such as aflatoxin, ochratoxin A, fumonisim and zearalenone (Paterson & Lima, 2010a). Of these four mycotoxins, research on aflatoxin has been extensively conducted (Paterson & Lima, 2010a; Patterson & Lima, 2010b; Kensler et al., 2011)

At the beginning of investigation, aflatoxin quantification was facilitated by their intense fluorescence under the ultraviolet (UV) light (Groopman et al., 2005). Later the isolation of purified aflatoxin metabolites with identical physical and chemical properties (Kensler et al., 2011) formed the core to the scientific research on aflatoxin to assess the possible hazards arising from the contamination of human food sources and finally to minimize the exposure through various preventive measures. In fact, the development of analytical methods of detecting and quantifying aflatoxin in the foods and feeds is significant, stimulated by the extensive research and collaborations (Kensler et al., 2011). As such, epidemiological and observational studies were possible to conduct to determine the association of aflatoxin ingestion with diseases in human population, especially with the incidence of hepatocellular carcinoma (HCC). Moroever, a better understanding on the mechanistic studies of aflatoxin toxicology and metabolism can be achieved from the development of methods for structural characterization and synthesis of the major aflatoxin metabolites (Groopman et al., 2005; Paterson & Lima, 2010a; Kensler et al., 2011). For instance, the isolation of aflatoxin biomarkers in human biological samples such as AFB₁-DNA adduct, serum AFB₁lysine adduct and other metabolites of AFB₁ in urine and faeces (Wang *et al.*, 1999; Mykkänen et al., 2005; Polychronaki et al., 2008) provide the tools to evaluate the molecular epidemiology of aflatoxin exposure of individuals within human population.

Having said that, the technology could be meaningless if human populations are still exposed to aflatoxin and there are no attempts to prevent the occurrence from continuing to happen. There are various measures developed for the control of aflatoxin contamination, which can range from physical, chemical and biological ways (El-Nezami *et al.*, 1998). Although these approaches could possibly prevent the "flow" of aflatoxin in the food chain, it is believed that humans are still at risk of being exposed as some contaminated foods might "escape" and persist in the food chain. This incessant event could potentially pose serious impact on human as aflatoxin is a dangerous toxicant and linked to the development of HCC (IARC, 1993; IARC 2002). People might recognize the appearance of fungus in the foodstuffs but aflatoxin is hardly recognisable by visual inspection as it is odourless, colourless, tasteless and invisible through the naked eyes.

Therefore, one of the preventive strategies is by minimizing and/or limiting humans' exposure to aflatoxins. The use of non-nutritional adsorbents such as activated carbon, hydrated sodium calcium alumino silicate, zeolite, bentonite and certain clays has shown to be beneficial in preventing absorption of aflatoxin in the gastrointestinal tract based on *in vitro* and *in vivo* experiments (Deli & Okan, 2006; Thieu & Pettersson, 2008; Gallo *et al.*, 2010). As an example, a clinical study using NovaSil clay was found to be an effective adsorbent of aflatoxin (Wang *et al.*, 2008). Regardless of the findings, their application for human intervention study is questionable as some could be dangerous and pose unwanted side effects to human health. Besides, increasing concern and demand by consumers for safe and high-quality foods have prompted research to find a better alternative.

Predicated upon that, the use of probiotic bacteria recently has been studied as one of the potential adsorbents of aflatoxin in the gastrointestinal tract. There are various studies from *in vitro* and animal which found the potential of certain probiotic lactic acid bacteria in reducing the bioavailability of aflatoxin (El-Nezami *et al.*, 1998; Hernandez-Mendoza *et al.*, 2009b; Peltonen *et al.*, 2000; Lahtinen *et al.*, 2004; Gratz et *al.*, 2006; Haskard *et al.*, 2000). In fact, probiotic bacteria have many beneficial health effects (Oelschlaeger, 2010), thus its use to counteract the toxicity of alftoxin could be further examined as one the preventive strategies.

1.2. Problem statement

Humans are vulnerable to many harmful exposures through ingestion, inhalation or even by contact (Paterson & Lima, 2010a). The contamination of human food resources by toxins, especially aflatoxins has become a burden to the public as people are not aware of this phenomenon. Due to that, many scientific studies have been conducted to find the roots of this epidemic problem. In one of many instances, Kensler *et al.* (2011) provided evidences on aflatoxin through extensive literature reviews and highlighted the pervasiveness of human exposure to aflatoxin particularly in Africa and Asia, where the prevalence of aflatoxin contamination in the food and agricultural products is higher. For example in Kenya, 55% of 658 maize products had aflatoxin level greater than the Kenyan regulatory limit of 2 ppb, 35% had levels >100 ppb and 7% had levels > 1000 ppb (Kensler *et al.*, 2011)

Malaysia has tropical climate that can favour the growth of fungi in the crops and many agricultural commodities. It is one of the several factors that contribute to the occurrence of aflatoxin. With poor food processing, storage and handling along with high temperature and humidity environments, the growth of aflatoxin-producing fungi on stored grains is favoured (Paterson & Lima, 2010b) and led to the production of aflatoxin. Even though fungal infection can be detected by visual inspection, aflatoxin is hardly detectable. As a result of the manifestation, research on aflatoxin has been studied extensively in Malaysia in order to extrapolate the extent of human exposure to this food contaminant. Besides, there are many articles published in the literatures on the prevalence of aflatoxin contamination in many foodstuffs and agricultural commodities in Malaysia (Abdullah *et al.*, 1998; Sulaiman *et al.*, 2007; Arzandeh *et al.*, 2010; Reddy & Salleh, 2010). For instance, Reddy *et al.* (2011) indicated that of 95 foods normally consumed by Malaysians, 69 (72.6%) were found positive for AFB₁ ranging from 0.53 to 15.33µg/kg.

Malaysia is reported to have high aflatoxin exposure ranging from 15 to 140 ng/kg body weight/day compared to other Southeast Asian countries such as Indonesia, Thailand and Philippines (Liu & Wu, 2010). This statistic is not something to be proud off as aflatoxin cases have been detected since 1980s [aflatoxicosis case in Perak (Lye *et al.*, 1995)] and a study by Zulhabri *et al.* (2009) found that Malaysian HCC patients had significantly high AFB₁-albumin adduct compared to the control subjects. In fact, these findings show how vulnerable humans to aflatoxin as some of the contaminated foods are detected with high aflatoxin levels. Sulaiman *et al.* (2007) revealed in accordance to the Malaysian Food Regulation 1985, the maximum permissible level of all mycological contaminants (aflatoxin and other mycotoxins) in all types of food is $35\mu g/kg$. Moreover, Leong *et al.* (2010) added that a limit of $15\mu/kg$ of total aflatoxins in groundnuts for processing have been established by the Malaysian Food Act 1983 and Food Regulations 1985.

One of the recent findings is the occurrence of aflatoxin in nuts, cereals, spices and herbs in Malaysia (Leong *et al.*, 2010; Arzandeh *et al.*, 2010; Sulaiman *et al.*, 2007). Aflatoxin exposure is harmful as this food contaminant poses serious health impact to humans as it is an integral for the pathogenesis of liver cancer (IARC, 2002). To date, many prevention strategies have been developed as seen through the *in vitro* and animal studies but its application in humans is very limited (Liu & Wu, 2010). Moreover, there is no direct approach to prevent human exposure to aflatoxin in the diets. Nonetheless, the use of adsorbents such as clay, activated carbon as well as certain probiotic bacteria can minimize the exposure rate in humans as they prevent aflatoxin absorption in the intestine (Kabak *et al.*, 2009; Hernandez-Mendoza *et al.*, El-Nezami *et al.*, 1998).

1.3. Significance of the study

Mycotoxins, especially aflatoxins have adverse effects on animals and humans; hence this research investigates the effectiveness of *Lactobacillus casei* Shirota (LcS) as a aflatoxin potential adsorbent to prevent aflatoxin absorption in the gastrointestinal tract. Even though there are many researches in Malaysia that found and/or detected aflatoxin in the foodstuffs, little is known about the extent of human exposure to aflatoxin. Generally, exposure assessment by measuring aflatoxin in food samples and extrapolating to calculate average intakes at the population level is of low reliability. The use of aflatoxin biomarkers such as serum AFB_1 -lysine adduct and urinary AFM_1 is beneficial as the formation of these biomarkers indicate a direct evidence of human oral ingestion to aflatoxin.

The detection of serum AFB₁-lysine adduct indicates aflatoxin exposure for the past 2 to 3 months, whereas for recent exposure (24 hours to 3-4 days), the urinary AFM₁ biomarker is used for the assessment (Williams *et al.*, 2004). Kensler *et al.* (2011) further explained that serum AFB₁-lysine adduct has been shown to correlate with AFB₁-DNA adduct (AFB₁-N⁷-guanine adduct), a promutagenic aflatoxin biomarker. It is a significant finding as formation of these adducts lies on the causal pathway of aflatoxin-induced hepatocellular carcinoma (Kensler *et al.*, 2011). Besides that, Zhu *et al.* (1987) have shown a positive correlation between dietary AFB₁ with the amount of AFM₁ detected in urine samples. Thus, with the available data on the occurrence of aflatoxin in the food and agricultural products, the expansion of metabolomic study through the research on aflatoxin biomarkers could be the bridge to establish a validated data for assessing human exposure to aflatoxin among individuals within a population. In fact, to my best knowledge, research on aflatoxin biomarkers in Malaysia is limited and still in its infancy stage and findings from this study could be used for a reference in the future.

As for the preventive measure for limiting/preventing human exposure to aflatoxin, several non-nutritional adsorbent such as activated carbon and clay have shown to be a good barrier for preventing aflatoxin absorption in small intestine. It is evident by Wang *et al.* (2008) as an intervention using clay reduces aflatoxin biomarkers in a population with high rate of aflatoxin contamination in the foodstuffs. Nevertheless, more alternative measures should be carried out and the use probiotic bacteria were found to be beneficial. Several *in vitro* and animal studies showed the ability of probiotic bacteria to adsorb aflatoxin molecules to their bacterial cell wall, thereby prevent aflatoxin absorption in the small intestine (El-Nezami *et al.*, 1998; Hernandez-Mendoza *et al.*, 2009b; Peltonen *et al.*, 2000; Lahtinen *et al.*, 2004; Gratz et *al.*, 2006; Haskard *et al.*, 2000). Of great significance is probiotic bacteria have many beneficial health effects as they can enhance immunity system, nourish intestinal microflora and improve gut-barrier function (Isolauri, 2001; Oelschlager, 2010). In fact, this research can add new knowledge on the potential ability of probiotic bacteria as an adsorbent of aflatoxin.

1.4. Hypothesis

This thesis comprised of two hypotheses. The main hypothesis is *Lactobacillus casei* Shirota strain (LcS) reduces the excretion and circulation of aflatoxin metabolites in urine and blood, whereas the working hypothesis is adults lack of knowledge on food contamination with fungi and aflatoxins.

1.5. Objective

1.5.1. General objective

To investigate the level of aflatoxin biomarkers in human blood and urine samples and to study the effectiveness of *Lactobacillus casei* Shirota strain (LcS) as a potential adsorbent of aflatoxin.

1.5.2. Specific objectives

- i. To determine the determinant of adults' knowledge of fungal and aflatoxin contamination in the diets among the subjects recruited during the screening stage.
- ii. To determine the level of aflatoxin M_1 (AFM₁) in urine samples and its association with food consumption.
- iii. To validate the UHPLC method for quantification of urinary AFM₁.
- iv. To determine the effectiveness of fermented milk containing LcS in reducing the level of aflatoxin biomarkers in human blood and urine samples among the subjects exposed to aflatoxin.
- v. To simulate human digestion in order to assess aflatoxin binding activity of LcS based on the bioaccessibility of aflatoxin.

1.6. Flow chart of the study

A screening study involved 160 subjects from a faculty in UPM

Subjects received a questionnaire to assess their knowledge on fungal and aflatoxin contamination in the diets. The determinants of subjects' knowledge were also investigated based on the socio-demographic characteristic (Chapter 3) Subjects also provided morning urine sample for the analysis of urinary AFM₁ using ELISA and to find its association with food consumption gathered from 37-food items FFQ. Risk assessment of aflatoxin was also assessed (Chapter 4)

Seventy-one subjects (n=71) were recruited based on the criteria assessed during the screening stage for an intervention study. An optimized UHPLC method was used to analyse urinary AFM₁ (Chapter 5 and 6)

A 4-week intervention study was carried out to investigate the effectiveness of fermented milk drink containing LcS in reducing aflatoxin biomarkers namely serum AFB₁-lysine adduct and urinary AFM₁ (Chapter 6)

An *in vitro* simulation of human digestion under fed condition was carried out to investigate LcS as a potential aflatoxin adsorbent (cultured LcS and probiotic drink used during the fermentation) in reducing AFB₁ bioaccessibility in AFB₁-spiked peanut samples (Chapter 7)

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