Universidade Estadual de Londrina

ANGÉLICA TIEME ISHIKAWA

AVALIAÇÃO DA EXPOSIÇÃO HUMANA À AFLATOXINA B_1 E M_1 EM LACTANTES E INFANTES E EFEITO IMUNOTOXICOPATOLÓGICO DE AFLATOXINA B_1 EM CAMUNDONGO C57Bl/6

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Este trabalho foi realizado na Universidade Estadual de Londrina e *Food Hygiene Laboratory, Faculty of Agriculture, Kagawa University* - Japão, sob a orientação da Drª. Eiko Nakagawa Itano, co-orientação de Drª. Elisa Yoko Hirooka e Dr. Osamu Kawamura (exterior), com o apoio da Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Rede Nanobiotec, Fundação Araucária e PROPPG/UEL.

DECLARAÇÃO DE REALIZAÇÃO DE TRABALHO

Eu, ANGÉLICA TIEME ISHIKAWA, autora do trabalho de Tese intitulado "Avaliação da exposição humana à aflatoxina B_1 e M_1 em lactantes e infantes e efeito imunotoxicopatológico de aflatoxina B_1 em camundongos C57Bl/6", declaro que realizei praticamente todo trabalho relatado nesta tese e que teve colaboração de Profa. Dra. Eiko Nakagawa Itano do Laboratório de Imunologia Aplicada, CCB/UEL, como orientadora; da Profa. Dra. Elisa Yoko Hirooka do laboratório de Ciências de Alimentos, CCA/UEL, como co-orientadora; do Prof. Dr. Osamu Kawamura de Kagawa University, Japão, como co-orientador no exterior referente à pesquisa humana; do Prof. Dr. J. Scott Weese da University of Guelph, Canadá, pela colaboração no sequenciamento de DNA na plataforma Illumina MiSeq referente a microbiota bacteriana em camundongos; do Dr. Marcio C. Costa, pos-Doc do laboratório de Virologia, CCA/UEL na interpretação dos dados da comunidade bacteriana intestinal e na revisão do manuscrito 3; da Profa. Dra. Ana Paula F. L. Bracarense do laboratório de Anatomia Patológica na interpretação dos dados histológicos. A Dra. Cássia R. Takabayashi-Yamashita contribuiu no preparo do manuscrito 1 e 2. A Profa. Dra. Elisabete Y. S. Ono e Msc. Fernando L. Sanches contribuíram no preparo do manuscrito 2. O Dr. Wagner E. Risso auxiliou no tratamento de camundongos com aflatoxina B_1 via gavagem. A Prof. Dra. Karina Keller auxiliou na realização das provas bioquímicas de função hepática. A Aparecida D. Malvezi contribuiu com as contagens de células da medula óssea. A Msc. Bruna Santos auxiliou na extração de DNA bacteriano em amostras de fezes de camundongos. A Dra. Paula L. A. e Silva e Msc. João Paulo Assolini colaboraram em algumas etapas finais de coleta e análise de amostras biológicas de camundongos. O Msc. Artur K. Bagatin auxiliou na realização dos testes envolvendo aflatoxina M_1. Declaro também que Msc. Cíntia L. Handa, Msc. Fernando S. de Lima, Felipe P. Fracalossi, Dra. Fabiana Rigobello, Thais D. Alexandrino, Cátia L. Yokoyama, Pamela L. R. Chagas ajudaram na obtenção e/ou transporte de amostras de leite materno; e o Dr. Ismael R. Amador liofilizou as amostras de leite materno. Assim como, Claúdia Y. Akagi, Dra. Cássia R. T. Yamashita, Msc. Daniele C. Honorato, Msc. Cíntia L. Handa, Msc. Fernando L. Sanches, Livia Medici, Bianca Dorana, Tawane Arduan, Gabriele Oliveira, Msc. Luiz Fernando Veríssimo auxiliaram no dia da eutanásia dos camundongos.

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A mente que se abre a uma nova ideia jamais
voltará ao seu tamanho original

Albert Einstein

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RESUMO

A exposição humana e animal às aflatoxinas (AFs), por meio de alimentos e rações contaminados, é um problema mundial devido ao seu potente efeito carcinogênico, hepatotóxico e imunossupressivo. A identificação do padrão de dieta, frequência de consumo, bem como determinação de níveis de metabólitos de AFs, como por exemplo aflatoxina M_1 (AFM_1), permitem fazer predições das relações entre dieta e saúde humana. Dessa forma, considerando a maior vulnerabilidade de infantes às micotoxinas, um questionário de frequência de consumo alimentar foi aplicado em mães lactantes (n = 93, Norte do Estado do Paraná, Brasil), visando avaliar o consumo de alimentos comumente contaminados com aflatoxina B_1 (AFB_1) e AFM_1, assim como foi estimada a exposição de infantes à AFM_1 em amostras de leite materno (BM) e leite em pó infantil (IPM) comercial. A média de consumo por mães lactantes foi de 2,73 g de milho/pessoa/dia e 0,79 g de amendoim/pessoa/dia, consumo considerado abaixo da média da América Latina. A extração de AFM_1 em BM (n = 94) e IPM (n = 16) foi realizada em coluna de imunoafinidade (CIA) *homemade* e à contaminação avaliada por Cromatografia Liquida de Alta Eficiência (CLAE) (BM, LOD: 0,004 ng/g, LOQ: 0,021 ng/g; IPM, LOD: 0,003 ng/g, LOQ: 0,016 ng/g). A AFM_1 foi detectada em 5,3% e 43,8% das amostras de BM e IPM, com níveis médios de 0,003 ng/g e 0,011 ng/g, respectivamente. Todas as amostras de IPM apresentaram níveis de AFM_1 abaixo daqueles estabelecidos pela legislação Brasileira (5 ng/g). Consequentemente, a ingestão diária estimada (EDI) de AFM_1 por infantes (0 a 12 meses) apresentaram valores baixos (0,019 a 0,347 ng/kg peso corpóreo/dia). Em paralelo, os efeitos imunotoxicopatológicos de AFB_1 foram investigados em camundongos C57Bl/6 (n = 5 cada grupo) utilizando uma única dose subclínica. (44, 442 e 663 µg/kg de peso corpóreo, p. c.) administrado via gavagem. Cinco dias após a exposição de AFB_1, os seguintes parâmetros foram avaliados: a) função hepática por meio de análise de níveis séricos bioquímicos (ALT, γ-GT, proteína total) e histopatologia do fígado; b) resposta linfoproliferativa de células de baço aos mitógenos concanavalina A (ConA) e lipopolissacarídeo (LPS) por teste de MTT, níveis de citocinas hepáticas (IL-4, IFN-γ e IL-17) por ELISA e contagem de células da medula óssea e, c) possível interferência de AFB_1 na comunidade bacteriana fecal pelo sequenciamento em Ilumina MiSeq. Todos os animais experimentais tratados mostraram alterações histológicas no fígado, porém com score significativo apenas na dose de 663 µg de AFB_1/kg de p. c. e nesta dose foi detectado *up*-regulação significativa de níveis de IL-4 e IFN-γ em fígado (p < 0,05), todavia os parâmetros séricos bioquímicos permaneceram normais. Nas doses de 442 e 663 µg/kg de p. c. houve uma supressão significativa na resposta linfoproliferativa a ConA, e aumento na contagem de leucócitos e neutrófilos, e na dose de 663 µg/kg de p. c. aumento de eritrócitos e megacariócitos na medula óssea. O tratamento com 663 µg de AFB_1/kg de p. c. aumentou a abundância relativa da família de bactérias Lachonospiraceae (p < 0,05). Pelos resultados obtidos concluímos que as mães lactantes no Norte do Estado do Paraná apresentam menor consumo de alimentos comumente contaminados com AFB_1 do que o consumo médio da América Latina e que a exposição de infantes à AFM_1 na região é baixa. Adicionalmente, uma única dose oral subclínica de AFB_1 pode induzir lesões no fígado, imunomodulação e possível mudança na microbiota intestinal em camundongos C57BL/6. O estudo pode contribuir para melhor entendimento da necessidade do contínuo controle da

qualidade de ração e alimento para minimizar o risco à saúde, especialmente a dos infantes. Além disso, os efeitos sistêmicos de doses subclínicas de AFB_1 devem ser explorados para maior compreensão da ação desta micotoxina, principalmente na avaliação da composição da microbiota intestinal.

Palavras-chaves: Micotoxina. Dieta. Infante. Imunomodulação. Microbiota intestinal.

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ABSTRACT

The human and animal exposure to aflatoxins (AFs), through contaminated food and feed, is a worldwide problem due to its potent carcinogenic, hepatotoxic and immunosuppressive effects. The dietary pattern identification, consumption frequency, as well as determination of AFs metabolites levels such as aflatoxin M_1 (AFM_1), allows to make predictions of the relationship between diet and health. Thus, considering the increased vulnerability of infants to mycotoxins, a food frequency questionnaire was applied to lactating mothers (n = 93, North of Paraná State, Brazil) to evaluate the consumption of foods commonly contaminated with aflatoxin B_1 (AFB_1) and AFM_1, and it was estimated exposure of infants to AFM_1 in breast milk (BM) and commercial infant powdered milk (IPM) samples. The average consumption by lactating mothers was 2.73 g of corn/person/day and 0.79 g of peanut/person/day, consumption considered below the average for Latin America. The extraction of AFM_1 in BM (n = 94) and IPM (n = 16) were performed in homemade immunoaffinity column (IAC) and evaluated concerning AFM_1 contamination by High Performance Liquid Chromatography (HPLC) (BM, LOD: 0.004 ng/g, LOQ: 0.021 ng/g; IPM, LOD: 0.003 ng/g; LOQ: 0.016 ng/g). The AFM_1 was detected in 5.3% and 43.8% of IPM and BM samples, with mean levels of 0.003 ng/g and 0.011 ng/g, respectively. All IPM samples showed AFM_1 levels below those established by the Brazilian legislation (5 ng/g). Consequently, the estimated daily intake (EDI) of AFM_1 for infants (0 to 12 months) showed low values (from 0.019 to 0.347 ng/ kg body weight/day). In parallel, immunotoxicpathological effects of AFB_1 were investigated in C57Bl/6 mice (n = 5 each group) using a single subclinical dose (44, 442 and 663 μg/kg body weight, b. w.) administered by gavage. Five days after exposure to AFB_1, the following parameters were evaluated: a) liver function through biochemical analysis of serum levels (ALT, γ-GT, total protein), liver histopathology; b) splenic lymphoproliferative responses to the concanavalin A (ConA) and lipopolysaccharide (LPS) mitogens by MTT assay, liver cytokines levels (IL-4, IFN-γ and IL-17) by ELISA, and bone marrow cell counts and c) possible AFB_1 interference in the fecal microbiota population by Ilumina MiSeq sequencer. All treated experimental animals showed histopathological changes in the liver, but with a significant score only at the dose of 663 μg of AFB_1/kg b. w. and at same dose was detected a significant up-regulation of IL-4 and IFN-γ levels in the liver ($p < 0.05$), but the serum biochemical parameters remained normal. At doses of 442 and 663 μg of AFB_1/kg b. w., there was a significant suppression in the lymphoproliferative response to ConA, and an increase in leukocytes, neutrophils, and at dose of 663 μg of AFB_1/kg b. w. increase erythrocytes and megakaryocytes in the bone marrow. Treatment with 663 ug of AFB_1/kg b. w. increased the relative abundance of bacteria Lachonospiraceae family ($p < 0.05$). We concluded that lactating mothers in Northern of Paraná State have lower consumption of foods commonly contaminated with AFB_1 than the average consumption in Latin America and infants exposure to AFM_1 is low in the region. Additionally, a single oral subclinical dose of AFB_1 may induce liver damage, immunomodulation and possible changes in the intestinal microbiota in C57Bl/6. The study can contribute to better understanding of the need to continuous food and feed quality controlling to minimize health risk, especially to infants. Furthermore, the understanding of systemic effects of subclinical doses of AFB_1 should be exploited for a better comprehension of mycotoxin action, particularly in the evaluation of intestinal microbiota composition.

Key words: Mycotoxin. Diet. Infant. Immunomodulation. intestinal microbiota.

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LISTA DE ABREVIATURAS E SIGLAS

µg	Micrograma, microgram
µL	Microlitro, microliter
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFs	Aflatoxins
ALARA	As Low As Reasonably Achievable
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
ANVISA	Agência Nacional de Vigilância Sanitária
AST	Aspartate Aminotransferase
B.W.	Body weight
BM	Human breast milk
BMDL10	Benchmark Dose Limit for a 10% increase cancer incidence
CD14	Cluster of Differentiation 14
CLAE	Cromatografia Líquida de Alta Eficiência
ConA	Concanavalin-A
CYP	Citocromo P 450
dL	Decilitro, deciliter
DMSO	Dimethyl Sulfoxide
DNA	Desoxyribonucleic Acid
EDI	Ingestão Diária Estimada
EFSA	The European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
g	Grama, gram
GALTs	Gut-Associated Lymphoid Tissues
GST	Glutationa-S-Transferase
h	Hora, hour

HE	Hematoxylin-Eosin
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HSFM	Hybridoma Serum Free Medium
HTLV1	Human T Lymphotropic Virus Type 1
HTLV2	Human T Lymphotropic Virus Type 2
IAC	Immunoaffinity column
IARC	International Agency for Research on Cancer
IC50	Half Maximal Inhibitory Concentration
IFN-γ	Interferon γ
IgA	Immunoglobulin A
IL-17	Interleukin 17
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin-6
IL-β	Interleukin β
IPM	Infant powdered milk
kg	Quiilograma
LOD	Limit of detection
LOQ	Limit of quantification
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
mL	Mililitro, milliliter
mm^3	Milimetro cúbico, cubic milimeter
MOE	Margin of exposure
MTT	3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide
Myd88	Myeloid Differentiation 88
n.d.	Not Detectable
NK	Natural Killer
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
OMS	Organização Mundial da Saúde
PBS	Phosphate Buffer Saline

PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
pg	Picograma, picogram
PI	Proliferation Index
PTDI	Provisional Tolerable Daily Intake
PTWI	Provisional Tolerable Weekly Intake
rpm	Rotation per minute
RPMI 1640	Roswell Park Memorial Institute 1640
rRNA	Ribosomal Ribonucleic Acid
RSD	Relative Standard Deviation
$TCD4^+$	T helper lymphocyte $CD4^+$
TD_{50}	Median Toxic Dose
Th1	T helper Type 1
Th17	T helper Type 17
Th2	T helper Type 2
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNFα	Tumor Necrosis Factor α
γ-GT	γ Glutamyl Transpeptidase

SUMÁRIO

1 INTRODUÇÃO

1.1 Leite materno

O leite materno é fundamental para saúde da criança pela disponibilidade de nutrientes e substâncias imunoativas. A amamentação exclusiva nos primeiros seis meses de vida favorece a relação afetiva mãe-filho, garante o melhor crescimento e desenvolvimento do bebê do ponto de vista cognitivo e psicomotor, proteção contra infecções gastrointestinal, respiratória e urinária, entre outros benefícios (BRASIL, 2010; LEVY, BERTOLO, 2012). O início da amamentação na primeira hora após o nascimento protege o recém-nascido contra infecções e reduz sua mortalidade. O leite materno é uma importante fonte de energia e nutrientes para crianças de 6 a 23 meses, podendo fornecer 50% ou mais de energia necessária a um infante de 6 a 12 meses, e aproximadamente 33% para crianças de 12 a 24 meses (WHO, 2014).

Em estudo realizado pelo Ministério da Saúde sobre aleitamento materno nas capitais brasileiras e no Distrito Federal, considerando parâmetro da Organização Mundial de Saúde (OMS) foi observado que o aleitamento na primeira hora de vida apresentou situação considerada boa (50 – 89%). Em relação ao Aleitamento Materno Exclusivo em menores de 6 meses, 23 capitais ainda se encontram em situação ruim (12 – 49%) e apenas 4 em boa situação (50 – 89%), enquanto a duração do aleitamento materno no Brasil ainda é considerada baixa (0 – 17 meses). A OMS recomenda que os alimentos complementares sejam oferecidos a partir do sexto mês de vida. No Brasil, a introdução do consumo de outros leites é precoce, com 18% das crianças já recebendo outros tipos de leites no primeiro mês de vida, com tendência crescente em faixas etárias subsequentes, chegando a 48,8% entre 120 e 180 dias (BRASIL, 2005; BRASIL, 2009a).

Em algumas situações pode haver restrição médica ao aleitamento com substituição parcial ou total do leite materno. O leite materno proveniente de mães usuárias de medicamentos e drogas, com infecção herpética da mama, varicela, doença de chagas, abcesso mamário e infectadas por *Human T Lymphotropic Virus Type 1* (HTLV1), *Human T Lymphotropic Virus Type 2* (HTLV2) e *Human Immunodeficiency Virus* (HIV), não é recomendado para o aleitamento (BRASIL, 2009b; LEVY, BERTOLO, 2012). Dessa forma, a fórmula infantil pode ser utilizada como substituto do leite materno, sendo que o leite materno e a fórmula infantil podem ser veículos de diversos contaminantes, entre eles as micotoxinas.

1.2 Aflatoxina B_1 e M_1

Em 1962, o termo micotoxina foi designado com a morte de aproximadamente 100.000 perus que consumiram ração contendo torta de amendoim contaminado com aflatoxina. A possibilidade de outros metabólitos secundários fúngicos causarem a morte sensibilizaram os cientistas, e o período entre 1960 e 1975 ficou conhecido como *Mycotoxin Gold Rush*. Cerca de 300 a 400 compostos são reconhecidos como micotoxinas, sendo que doze grupos recebem atenção devido a ameaça à saúde humana e animal (ATANDA *et al.,* 2012; BENNETT, KLICH, 2003). As micotoxinas são capazes de induzirem efeito carcinogênico, hepatotóxico, imunossupressivo, genotóxico, mutagênico, entre outros (FROMME *et al.,* 2016). A exposição humana à micotoxinas ocorre diretamente por meio da ingestão de produtos agrícolas contaminados (cereais, frutas, especiarias, entre outros) ou indiretamente pelo consumo de produtos de origem animal (ovos, leite, carne, entre outros) preparados ou obtidos de animais que consumiram alimentos contaminados com micotoxina (FLORES-FLORES *et al*., 2015; PLEADIN *et al*., 2015)

Entre as micotoxinas, as aflatoxinas (AFs) representam um grande problema para saúde e economia no mundo, como já evidenciado por sua frequente detecção em alimentos e *commodities* agrícolas (LIU *et al*., 2013). O grupo abrange aproximadamente 20 metabólitos fúngicos produzidos principalmente por *Aspergillus flavus* e *A. parasiticus* (VARGA *et al*., 2011), mas apenas quatro desses análogos (B_1, B_2, G_1 e G_2) são considerados como contaminantes alimentares significativo (BELOGLAZOVA; EREMIN, 2015). A susceptibilidade do produto alimentar à contaminação fúngica ocorre durante a pré-colheita, transporte, e armazenamento de alimentos. Dessa forma, as AFs tem sido detectadas em várias *commodities* agrícolas, mas a contaminação mais pronunciada é relatada em milho, amendoim, sementes de algodão e fruto seco (IARC, 2002).

Entre os diferentes análogos de AFs, a aflatoxina B_1 (AFB_1) é a mais tóxica, considerando que a extensão da toxicidade depende de como o fígado é afetado. A Agência Internacional de Pesquisas em Câncer (IARC) classifica AFB_1 no Grupo 1, i.e., carcinogênico ao homem (IARC, 2002). As AFs são altamente lipossolúveis facilitando a sua absorção pelo trato gastrointestinal e respiratório, alcançam a corrente sanguínea, e posteriormente diferentes tecidos e órgãos (IARC, 2002). A aflatoxina requer ativação metabólica para o intermediário eletrofilico para exercer atividade carcinogênica. Esta espécie pode reagir e modificar o DNA, levando à formação de lesão pró-mutagênica que resulta na ativação de proto-oncogenes e inativação de genes supressores de tumor. AFB_1 sofre oxidação de dois elétrons inicialmente por citocromo P450 (CYP) 1A2 e 3A4, produzindo aflatoxina B_1- 8,9-óxido. Este epóxido reage com o átomo de guanina N7 para formar

um aducto de DNA pró-mutagênico (aflatoxina-N7-guanina). Além da formação de 8,9-óxido, a oxidação pela CYP 1A2 produz a aflatoxina M_1 (AFM_1), um composto monohidroxilado de AFB_1, considerado menos tóxico (KENSLER *et al.,* 2003; BBOSA et al., 2013).

A AFB_1 além de ser um potente hepatocarcinogênico, mutagênico, teratogênico, também apresenta propriedade imunossupressiva (SHARMA *et al*., 2011). Os efeitos imunotóxicos de AFB_1 estão relacionados com a interferência na imunidade mediada por células com redução do número de linfócitos circulantes, inibição da blastogênese de linfócitos em animais de várias espécies, alteração na atividade de células *Natural Killer*, expressão de citocinas, e possível capacidade de alteração na função de macrófagos. Além disso, a AFB_1 altera a capacidade de células da medula óssea em formar colônias mielóides e eritróides (BIANCO et al., 2012, GREINER et al., 2013).

A intoxicação resultante da ingestão de AFs, a aflatoxicose, pode ocorrer em seres humanos e em outros animais de forma aguda ou crônica. A toxicidade pode ser influenciada por fatores ambientais, idade, estado de saúde e nutricional do organismo, dose e duração de exposição à micotoxina (FDA, 2016). Casos de aflatoxicose aguda em seres humanos já foram relatados em populações de países em desenvolvimento, como os da África e Ásia (MWANDA, OTIENO, OMONGE, 2005; SAMUEL et al., 2009; FAO, 2014), com apresentação de sérias alterações no metabolismo, e consequentemente a morte. As espécies de animais respondem diferentemente quanto à toxicidade letal de AFB_1 devido as diferenças de sua biotransformação, variando desde animais extremamente susceptíveis (porco, pato, coelho) até espécies resistentes, como por exemplo camundongos, macaco e galinha (BBOSA et al., 2013). Os efeitos crônicos resultam da ingestão baixa a moderada de AFs a longo prazo, com efeitos subclínicos de difícil reconhecimento (FDA, 2016).

Os metabólitos de AFB_1 podem ser biotransformados e usados como evidência da exposição a aflatoxina e risco de carcinoma hepatocelular, como presença de aductos, aflatoxina-albumina, aflatoxina-N7-guanina, aflatoxina-lisina e AFM_1 (ROMERO *et al.,* 2009). A quantidade de AFM_1 que é excretada no leite como porcentagem de AFB_1 é de 1-2% em animais. A AFM_1 pode ser detectada no leite após a primeira ingestão de AFB_1 em 12-24h, alcançando altos níveis de contaminação em poucos dias. Quando a ingestão de AFB_1 cessa, a concentração de AFM_1 cai para níveis não detectados após 72h (ZINEDINE *et al*., 2007; SIDDAPPA *et al*., 2012). AFM_1 é menos carcinogênica ou mutagênica quando comparada à AFB_1, apresentando de 2-10% de carcinogenicidade (KENSLER *et al.,* 2003; ZINEDINE *et al*., 2007), sendo classificada no grupo 2B, *i. e*., como possível carcinógeno aos humanos (IARC, 1993).

A presença de AFM_1 em leite e produtos derivados de leite é uma preocupação mundial, uma vez que estes produtos são consumidos por toda faixa etária da população, incluindo infantes (EC, 2010). Dados sugerem que crianças são mais vulneráveis à aflatoxina do que adultos devido à alta taxa metabólica, baixo peso corpóreo, vias metabólicas imaturas e desenvolvimento incompleto de tecidos e órgãos. Por causa do perigo da natureza da toxina, muitos países têm definido ou proposto regulamento para níveis máximos de AFM_1 no leite e seus derivados. As variações nas regulamentações em diferentes países além de levar em consideração a saúde, sendo também ponderadas questões políticas e econômicas (SIDDAPPA *et al*., 2012; SIGNORINI *et al*., 2012). A Comunidade Europeia estabeleceu níveis máximos de 0,05 µg/kg de AFM_1 em leite fluido; 0,5 µg/kg em leite em pó; e 0,025 µg/kg para fórmula infantil (EC, 2010). No Brasil, a Agência Nacional de Vigilância Sanitária (ANVISA) não estabeleceu legislação de AFM_1 para produtos infantis (ANVISA, 2011).

Desta forma, o presente estudo objetivou avaliar a frequência de consumo de alimentos que são possíveis fontes de contaminação por aflatoxina B_1 e M_1 por mães lactantes, assim como avaliar o grau de exposição de infantes à aflatoxina M_1, cujo parâmetro é de grande importância na avaliação de risco. Além disso, existem poucos relatos na literatura sobre a exposição de dose subclínica única de aflatoxina B_1, somente trabalhos relatando a exposição crônica e aguda. Sendo assim, foram avaliados os efeitos sistêmicos de dose subclínica única de aflatoxina B_1 em camundongos C57Bl/6, com ênfase em parâmetros bioquímicos, sistema imune, histopatológicos e composição da microbiota intestinal.

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3 OBJETIVO

3.1 Objetivo geral

Avaliar a exposição de mães lactantes e infantes a aflatoxina B_1 (AFB_1) e M_1 (AFM_1) e avaliar o efeito imunotoxicopatológico de AFB_1 em camundongos C57Bl/6.

3.2 Objetivos específicos

i. Avaliar a frequência de consumo de alimentos comumente contaminados com AFB_1 e M_1 por mães lactantes no Norte do Estado do Paraná, Brasil, por meio de aplicação de questionário.
ii. Padronizar método de extração de AFM_1 em amostras de leite materno e leite em pó infantil utilizando coluna de imunoafinidade *homemade*
iii. Obter amostras de leite materno e determinar níveis de AFM_1 por Cromatografia Líquida de Alta Eficiência;
iv. Determinar níveis de AFM_1 em amostras de leite em pó infantil por Cromatografia Líquida de Alta Eficiência;
v. Calcular a Ingestão Estimada Diária para infantes (0 a 12 meses) do gênero masculino e feminino.
i. Determinar peso corpóreo e peso de órgãos de camundongos expostos a diferentes doses subclínicas de AFB_1 por via oral.
ii. Avaliar a função hepática em camundongos expostos a diferentes doses subclínicas de AFB_1 por via oral.
iii. Determinar o perfil hepático das citocinas (IL-4, INF- γ, IL-17) em camundongos expostos a diferentes doses subclínicas de AFB_1 por via oral.
iv. Avaliar o perfil histopatológico do fígado de camundongos expostos a diferentes doses subclínicas de AFB_1 por via oral.
v. Avaliar a resposta linfoproliferativa a mitógenos (concanavalina A e lipopolissacarídeo) em camundongos expostos a diferentes doses subclínicas de AFB_1 por via oral.
vi. Avaliar o efeito de exposição de camundongos a diferentes doses subclínicas de AFB_1 por via oral sobre as células de medula óssea.
vii. Avaliar o efeito de exposição de camundongos a diferentes doses subclínicas de AFB_1 por via oral sobre a microbiota bacteriana intestinal.

4. FLUXOGRAMA EXPERIMENTAL

A Figura 1 apresenta o fluxograma referente ao procedimento experimental sobre o consumo de alimentos potencialmente contaminados com aflatoxina B_1 e M_1 por mães lactantes, avaliação da exposição de infantes a aflatoxina M_1 em leite materno e leite em pó infantil, e exposição a aflatoxina B_1 em camundongos C57Bl/6.

O detalhe de cada procedimento técnico foi apresentado na forma de quatro manuscritos.

- ✓ **Manuscrito 1:** Food frequency consumption and estimative of dietary exposure to aflatoxin B_1 and M_1 by lactating mothers in the North of Paraná State, Brazil

- ✓ **Manuscrito 2:** Exposure assessment of infants to aflatoxin M_1 through consumption of naturally contaminated milk

- ✓ **Manuscrito 3:** Effect of administration of single oral subclinical doses of aflatoxin B_1 in the liver and gut microbiota in C57Bl/6 mice

- ✓ **Manuscrito 4:** Effect of single oral subclinical doses of aflatoxin B_1 on bone marrow cells and lymphoproliferative response to mitogens in C57Bl/6 mice

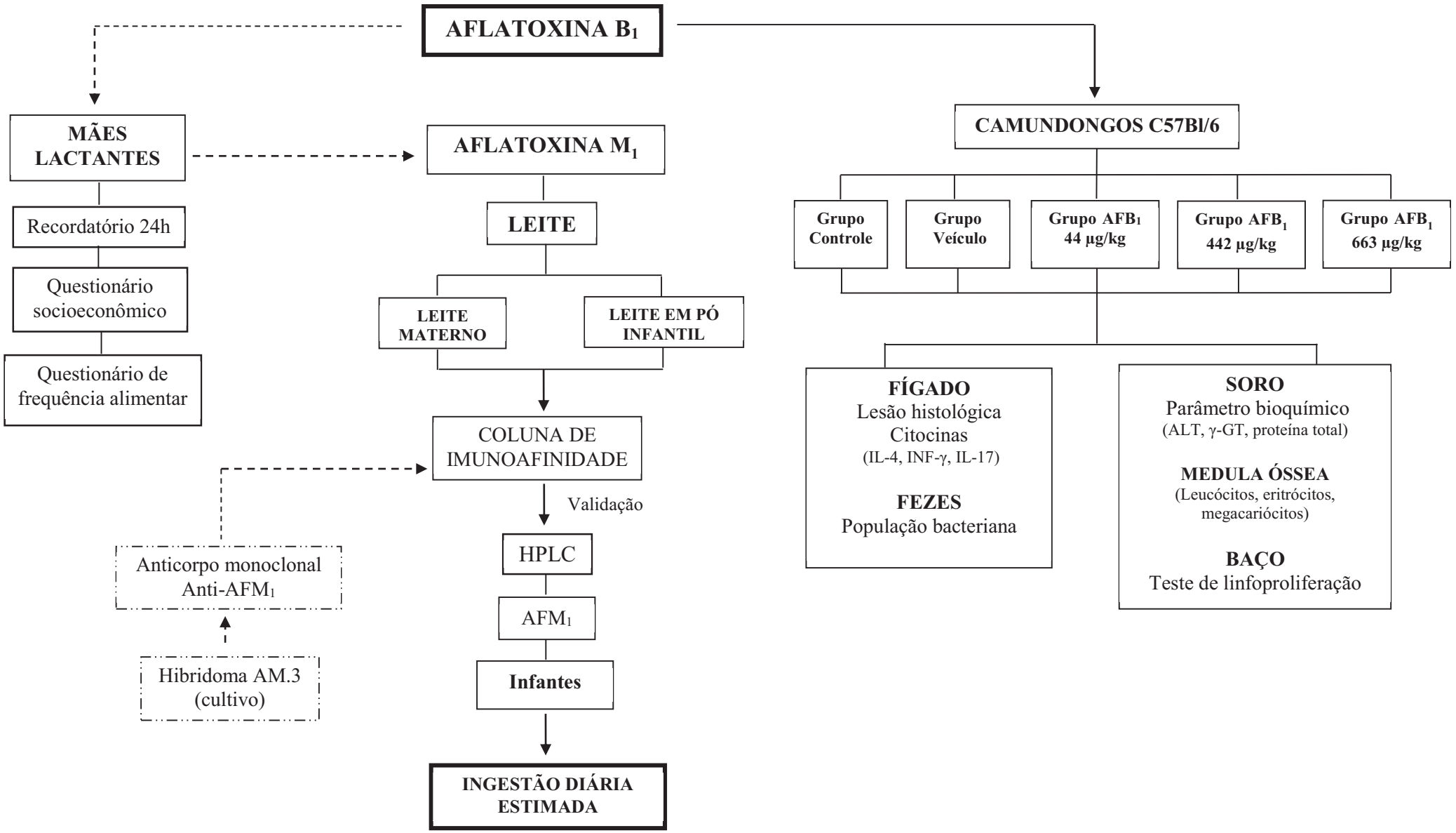

Figura 1. Fluxograma experimental sobre a exposição de mães lactantes, infantes e camundongos C57Bl/6 à aflatoxina B_1 e/ou M_1.

Manuscrito 1:

Food frequency consumption and dietary exposure to aflatoxin B_1 and M_1 by lactating mothers in the North of Paraná State, Brazil

Formatado nas normas do periódico Ciência e Agrotecnologia

5 MANUSCRITO 1: FOOD FREQUENCY CONSUMPTION AND ESTIMATIVE OF DIETARY EXPOSURE TO AFLATOXIN B_1 AND M_1 BY LACTATING MOTHERS IN THE NORTH OF PARANÁ STATE, BRAZIL

Frequência de consumo alimentar e exposição alimentar a aflatoxina B_1 e M_1 por mães lactantes no Norte do Estado do Paraná, Brasil

5.1 ABSTRACT

The human exposure to aflatoxins (AFs), mainly aflatoxin B_1 (AFB_1), represent a major problem for health due as a potent hepatocarcinogenic, mutagenic, teratogenic, also as immunosuppressive agent. These harmful health effects should be more impactful to newly born making it important the investigation of the food usually consumed by lactating mothers. The objective of this study was to evaluate the consumption frequency of food commonly contaminated with aflatoxin B_1 (AFB_1) and M_1 (AFM_1) and evaluate dietary exposure to AFs by lactating mothers in the North of Paraná State. A food frequency questionnaire was applied to lactating mothers (n = 93), from June to August of 2013 in the North of Paraná State, Brazil, and the consumption of foods commonly contaminated with AFB_1 and aflatoxin M_1 (AFM_1) was evaluated. The average of food consumption was 206.49 mL of raw milk/person/day, 2.73 g of corn and 0.79 g of peanut. The majority of lactating mothers (37.6%) consumed 400 mL/person/day of raw milk, 31.2% consumed 2.86 – 5.71 g of corn and 34.4% consumed 0.47 g of peanut. The consumption of corn and peanut by lactating mothers was low when compared to average food consumption of Latin American. Despite the low frequency of AFB_1 and AFM_1 contamination in corn and milk products marketed or produced in Paraná State, the Margin of Exposure (MOE) value for AFB_1 was similar for Latin America. Therefore, it is essential to know if the lactating mothers are consuming a significant amount of AFB_1-contaminated product that can lead to serious problems to their health, and consequently for infants.

Index terms: diet, mycotoxin, lactating mother, infant, exposure

RESUMO

A exposição humana a aflatoxinas (AFs), principalmente aflatoxina B_1 (AFB_1), representa um problema grave à saúde como potente agente hepatocarcinogênico, mutagênico, teratogênico, e também como agente imunossupressivo. Estes efeitos prejudiciais à saúde devem ser mais impactantes para recém-nascidos, tornando-se importante a investigação de alimentos contaminados comumente consumidos por mães lactantes. O objetivo deste estudo foi avaliar a frequência de consumo de alimentos comumente contaminados com aflatoxina B_1 (AFB_1) e M_1 (AFM_1) e avaliar a exposição a AFs por mães lactantes no Norte do Estado do Paraná. Um questionário de frequência alimentar foi aplicado em mães lactantes (n = 93), de junho a agosto de 2013 no norte do Paraná, Brasil, e o consumo de alimentos comumente contaminados com AFB_1 e aflatoxina M_1 (AFM_1) foi avaliado. A média de consumo alimentar foi 206,49 mL de leite/pessoa/dia, 2,73 g de milho e 0,79 g de amendoim. A maioria das mães lactantes (37,6%) consumiram 400 mL de leite/pessoa/dia, 31,2% consumiram 2,86-5,71 g de milho e 34,4% consumiram 0,47g de amendoim. O consumo de milho e amendoim pelas mães lactantes foi baixo quando comparado com a média de consumo da América latina. Apesar da baixa frequência de contaminação por AFB_1 e AFM_1 em milho e leite comercializados ou produzidos no estado do Paraná, o valor da Margem de Exposição (MOE) para AFB_1 em milho foi similar ao da América Latina. Portanto, é essencial saber se mães lactantes estão consumindo uma quantidade significativa de produtos contaminados com AFB_1 que podem levar a sérios problemas a sua saúde, e consequentemente para infantes.

Termos para indexação: Dieta, micotoxina, lactantes, infante, exposição.

5.2 INTRODUCTION

Diet and its components are considered as the most important risk factors for cancer worldwide (Gross-Steinmeyer and Eaton, 2012). The liver cancer is the fifth most common type of cancer worldwide and ranks third regarding global mortality. The factors responsible for the development of liver cancer are advanced age, sex steroids, hepatitis B and C, alcohol intake, smoking and aflatoxins (Kensler et al., 2003; Inca, 2015). Aflatoxins can be present in food, such as corn, peanuts, treenuts, rice, cottonseed, figs, dried foods, spices, and cocoa beans because of *Aspergillus* spp. contamination before and after harvest (EFSA, 2014).

There are four major aflatoxins namely aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2). However, AFB_1 is the most commonly occurring and has the greatest toxigenic potential. It has been considered a mutagenic, teratogenic and carcinogenic compound, classified as a Group 1, i.e., carcinogenic to humans (IARC, 2012; Jager et al., 2013). Aflatoxin M_1 (AFM_1) is monohydroxylated derivative of AFB_1 metabolized in liver by Cytochrome P450-associated enzymes in humans and animals, and then excreted primarily in the urine and less in the milk (Britzi et al., 2013). The International Agency for Research on Cancer classified AFM_1 in Group 2B, possibly carcinogenic to humans (IARC, 2002).

Infants are considered more susceptible to adverse effects of AFM_1 than adults, because of their low body weight, high metabolic rate and incomplete development of organs (Iarc, 2002). Consumption of food with high levels of aflatoxins may produce acute aflatoxicosis which in extreme cases may lead to death (Adejumo et al., 2013). In this way, concerning mycotoxin effects on human health, many countries set the maximum level of AFB_1 and AFM_1 in foodstuffs. In addition, frequency of food questionnaire allows identifying diet patterns, and permit to make predictions in the long term exploring the relationship among diet and health risk (Wall-Martínez et al., 2014). Based on the

importance of AFB_1 and AFM_1 risks for lactating mother and infant health, the aim of this study was to evaluate the frequency of consumption of food commonly contaminated with AFB_1 and AFM_1 by lactating mothers in the North of Paraná State.

5.3 MATERIAL AND METHODS

5.3.1 Lactating mothers

A total of 93 lactating mothers that agreed to participate in the study conducted in three hospitals in Londrina City - Brazil (Teaching Hospital of State University of Londrina, Evangelical Hospital and Municipal Maternity), from June to August 2013. The project was approved by the Human Ethics Committee of State University of Londrina (CEP/UEL, 159/2012). All volunteers were informed about the study protocol, and a written informed-consent agreement was signed.

5.3.2 Collection of socioeconomic and frequency of food consumption data

Socioeconomic data (age, working status, income) and frequency of food consumption of the foodstuffs that are commonly contaminated with AFB_1 (chili powder, paprika, nutmeg; Brazil nut; pistachios, almonds, hazelnuts; peanut; pé-de-moleque, paçoca; corn) and AFM_1 (raw milk; yogurt; cheese) were recorded by interview. “Pé-de-moleque” and “paçoca” are traditional Brazilian products derived from peanuts. The consumption data of products commonly contaminated with AFB_1 and AFM_1 was expressed in g/day or ml/day, and the frequency of consumption (%) was classified in: no consumption, daily (1 time or 2 times), weekly (1 or 2 times), weekly (3 or 4 times), every 15 days, or monthly (Table 1).

5.3.3 Average food consumption, dietary exposure estimation and margin of exposure to aflatoxin B_1 and M_1

Average food consumption was expressed in g or mL/person/day. AFM_1 dietary exposure (ng/kg body weight - b. w./day) for lactating mothers was calculated considering milk average consumption (206.49 mL/person/day), AFM_1 contamination in milk from Paraná State (Sassahara et al., 2005; Becker et al., 2010; Santos et al., 2014; Silva et al., 2015), and average body weight of lactating mothers (64.33 kg) (Formula 1). For AFB_1 dietary exposure, it was considered corn average consumption (2.73 g/person/day), and AFB_1 contamination in corn from Paraná State (Amaral et al., 2006; Moreno et al., 2009; Souza et al., 2013; Schmidt et al., 2015). AFB_1 is a genotoxic and carcinogenic compound, and therefore, Margin of exposure (MOE) should be estimated. MOE was calculated by dividing 95% lower confidence limit of the benchmark dose for a 10% increase in cancer incidence ($BMDL_{10}$) by dietary exposure (Formula 2, EFSA, 2007). In this study, $BMDL_{10}$ calculated by Benford et al. (2010a) was used, which is 250 ng/kg body weight/day.

$$\textbf{Dietary exposure } (\textbf{ng/kg b.w./day}) = \frac{\text{food intake x mean aflatoxin concentration}}{\text{average body weight}} \qquad (1)$$

$$\textbf{MOE} = \frac{\text{BMDL}_{10}}{\text{dietary exposure}} \qquad (2)$$

5.4 RESULTS AND DISCUSSION

An important practice is breastfeeding up to two years old or more, advantages such as optimal growth, development and health are ensured from exclusive breastfeeding for the first six months of infant's life (Brasil, 2009). Therefore, the diet of mother during breastfeeding should be as healthy as possible. The education level of mother is important

for the nutritional status of children, since the family's education level and their socioeconomic status have considerable effects about the way of life and eating habits of children (Silva et al., 2007). In this study, the socioeconomic status of lactating mothers showed variations in age, income and working status (Table 2). A total of 39 women (42%) were aged from 20 to 30 years old and 69% of them had an occupation. In addition, the most part of lactating mothers earned 1-3 minimum salary (55%). Families that belong to a lower economical class are more likely to consume foodstuffs contaminated with aflatoxins (Adejumo et al., 2013). Adejumo et al. (2013) reported that the socioeconomic status of the lactating mothers significantly influenced their dietary exposure and exposure risk of infants to AFM_1 in Nigeria. Mothers who coursed the primary school as the highest educational qualification presented a greater exposure risks to AFB_1 if compared to mothers who got higher qualifications; as well as traders had higher risks of dietary exposure to AFB_1 compared to bankers.

Concerning foodstuffs commonly contaminated with AFB_1, the most of lactating mothers did not consume chili powder, paprika, nutmeg (65.6%); Brazil nut (77.5%); and pistachios, almond, hazelnut (69.9%) (data not shown). The most often consumption of foodstuffs commonly contaminated with AFB_1 and AFM_1 is shown in Figure 1. A total of 34.4% of mothers consumed peanuts once a month (corresponding to 0.47 g/person/day) and 31.2% consumed corn weekly (1-2 times; 2.86 - 5.71 g/person/day). About food source contaminated with AFM_1, 37.6% of mothers consumed raw milk daily (2 times), and 25.8% and 22.6% consumed yogurt and cheese weekly (1-2 times), respectively. The average of food consumption was 0.79 g/person/day for peanut, 2.73 g/person/day for corn and 206.49 mL/person/day for raw milk (Table 3). These values are lower than the average food consumption for Latin American, 2.2 g of peanut/person/day, 64.8 g of corn/person/day

(JECFA, 2008); however, the average consumption of raw milk by lactating mothers (206.49 mL/person/day) was higher than Brazilian women (35.60 mL/person/day) (Brasil, 2010).

In Brazil, there are several reports on the occurrence of aflatoxins in food, but it has been reported mainly in corn and peanuts, which the largest producers of peanuts and corn in Brazil are São Paulo and Paraná State, respectively. In the study conducted by Jager et al. (2013) in São Paulo State, aflatoxins were assessed in peanut products, corn flour, corn white hominy, corn for popcorn, bean, fluid milk, powdered milk and cheese; and only peanut products samples were contaminated (0.056 - 36.7 μg/kg) above the limit established by the Brazilian legislation, which is 20.0 μg of AFs/kg (Table 3 – ANVISA, 2011). Similarly, high aflatoxin contamination in peanut in São Paulo State was reported in other studies (Shundo et al., 2010; Atayde et al., 2012; Imamura et al., 2015). Meanwhile, the AFB_1 contamination in corn and derivatives in North of Paraná State, ranged from not detected (n.d.) to 56 μg of AFB_1/kg, but the frequency of contamination was low (zero to 28.6%) (Sekiyama et al., 2005; Amaral et al., 2006; Moreno et al., 2009; Souza et al., 2013). Table 4 shows a review of aflatoxin B_1 occurrence in corn and derivatives in the Paraná State from 2005 to 2015.

The AFB_1 contamination in commodities provided to feed animals is a problem. Human exposition to mycotoxin can also occur through the consumption of products of animal origin, such as milk and eggs (Flores-Flores et al., 2015). Following ingestion of AFB_1 present in feedstuffs, it is metabolized in animal liver resulting in various metabolites, including AFM_1 that is excreted in milk. The AFM_1 contamination in milk purchased in markets or farms from Paraná State is shown in Table 4.

Considering the higher consumption of foodstuffs commonly contaminated with AFs was in corn and milk by lactating mothers, the dietary exposure of AFB_1 and AFM_1 was calculated (Table 3). In this study, the dietary exposure to AFM_1 in milk was 0.39 ng/kg b.w./day, and for AFB_1 in corn was 0.33 ng/kg b.w./day. According to Joint FAO/WHO

Expert Committee on Food Additives (JECFA), the average dietary intake of AFM_1 for adults is 3.5 ng/person/day and 0.3 - 0.5 ng/kg body weight/day for AFB_1 based on the Latin American diet (JECFA, 2001, 2008).

The MOE approach has been considered to be a useful pragmatic option for Risk Assessment of substances that are both genotoxic and carcinogenic, as it is AFB_1 (Leong et al., 2011). It takes into account the dietary exposure and the available data on the dose response relationship (Benford et al., 2010b). The magnitude of the MOE gives an indication of the level of concern, the larger MOE represents smaller potential risk by AFB_1 exposure. The European Food Safety Authority (EFSA) considered a MOE of 10,000 or higher of low concern from a public health point of view, which might be considered as a low priority for risk management actions (EFSA, 2005)

In this study, the MOE between a point of departure on the dose response for oral carcinogenicity in animal studies and estimates of human dietary exposure was 756. South America was considered a region with low exposure to AFB_1, presenting MOE value for population ranging from 833 to 500 (Benford et al., 2010a; 2010b). However, those MOEs values suggested that AFB_1 would be of public health concern. In Brazil, the annual incidence of liver cancer rate standardized by age per 100,000 inhabitants, it was 8.4 in Palmas city, 5.5 in Manaus and 5.3 in Recife; in the Paraná State did not have information about incidence of liver cancer (Inca, 2015). In Europe, hepatocellular carcinoma rates for females ranged from 1.7 to 4.0 per 100,000 per year (EFSA, 2007).

Furthermore, AFs as an agent that induces immunosuppression, it can contribute for increasing susceptibility to children infections or even interfering in immunization programs. Thus, it is important to know the aflatoxin contamination level in foodstuffs from Paraná State, and how often lactating mothers consume products produced/commercialized in the

Southern of Brazil. In addition, the study period was performed mostly in the winter season, it would be important to evaluate for a longer period.

5.5 CONCLUSIONS

The consumption of main food source of aflatoxin B_1 and M_1 in Brazil, such as corn and peanuts, by lactating mothers in Northern Paraná State was lower than the average food consumption of Latin America. Despite the low AFB_1 and AFM_1 contamination in corn and milk products marketed or produced in Paraná State, the MOE calculated for AFB_1 suggested that it would be a public health concern. Therefore, continuous monitoring should be assessed to obtain food quality that can reach the consumer as lactating mother, and consequently the infants.

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Table 1 Foodstuffs that can be possible sources of aflatoxin B_1 and M_1 contamination, and the representation of the portion in g or mL of these foods depending on the frequency consumption

Frequency of consumption	FOODSTUFFS				
	Chilli powder, Paprika, Nutmeg (g/day)	**Brazil nut; pistachios, almonds, hazelnut; corn or cheese (g/day)**	**Peanut (g/day)**	**Pé-de-moleque, paçoca (g/day)**	**Raw milk; yogurt (mL/day)**
Daily (1 time)	5.00	20.00	14.00	22.00	200.00
Daily (2 times)	10.00	40.00	28.00	44.00	400.00
Weekly (1-2 times)	0.71 – 1.43	2.86 – 5.71	2.00 – 4.0	3.14 – 6.29	28.57 – 57.14
Weekly (3-4 times)	2.14 – 2.86	8.57 – 11.43	6.00 – 8.0	9.43 – 12.57	85.71 – 114.28
Every15 days	0.33	1.33	0.93	1.47	13.33
Monthly	0.17	0.67	0.47	0.73	6.67

Table 2. Socieconomic status of lactating mothers (*n* = 93) from North of Paraná State, data collected from June to August of 2013.

SOCIOECONOMIC STATUS					
Age group (years)	**% (*n*)**	**Income (Salary*)**	**% (*n*)**	**Occupation status**	**% (*n*)**
< 20	16 (15)	< 1	4 (4)	Not occupation	31 (29)
20 – 30	42 (39)	1 – 3	55 (51)	Occupation	69 (64)
31 – 40	41(38)	4 – 6	24 (22)		
> 40	1 (1)	> 6	16 (15)		
		No answered	1 (1)		

* In 2013, 1 minimum salary was equivalent to R$ 678.00 (U$ 297.00)

Table 3. Average food consumption of foodstuffs commonly contaminated with AFB_1 and AFM_1, Tolerable maximum limit and dietary exposure

Foodstuff	Average Food Consumption (g or mL/person/day)		Mycotoxin		Aflatoxin exposure			
					Dietary*	MOE**	Dietary	MOE
	In this study	Literature		Maximum Levels[c] (µg/kg)	In this study		Literature	
Peanut	0.79	2.20[a]	AFB_1	20.00	0.33 ng/kg b.w./day	758	0.3-0.5 ng/kg b.w./day[a]	833 – 500[e]
Corn	2.73	64.80[a]						
Paprika		0.40[a]						
Chilli powder	0.11	N.A.[a]						
Nutmeg		N.A.[a]						
Brazil nut	0.07	0.10[a]						
Pistachio		0[a]		10.00				
Almond	0.87	0[a]						
Hazelnut		0[a]						
Raw Milk	206.49	35.60[b]	AFM_1	0.50	0.39 ng/kg b.w./day	-	3.5 ng/person/day[d]	-
Yogurt	94.00	11.00[b]		-				
Cheese	6.51	6.90[b]		2.50				

* Dietary exposure was calculated considering AFB_1 and AFM_1 mean contamination in corn and milk, respectively, from Paraná State (Table 4), and average body weight of lactating mothers (64.33 kg)

** Margin of exposure (MOE) was calculated based on $BMDL_{10}$ (250 ng/kg b. w. /day) divided by AFB_1 dietary exposure in corn by lactating mothers (0.5 ng/kg b.w./day)

[a] Data from Country Assignments to the 13 Proposed GEMS/Food Consumption Cluster Diet, k cluster - including Brazil (JECFA, 2008);

[b] Average food consumption of Brazilian women (Brasil, 2010);

[c] ANVISA, 2011;

[d] Dietary exposure in the Latin America diet (JECFA, 2001)

[e] Benford et al., 2010a

N.A.: not avaliable

Table 4. Occurrence of aflatoxin B_1 and M_1 in corn/derivatives and milk, respectively, in the Paraná State during 2005-2015

Products	Year/Crop	City/region	n_{total} / + (%)	Range (µg/kg)	Method	LOD (µg/kg)	Reference
AFB_1							
Corn meal and corn meal grits	2003-2004	Markets of Maringá and Marialva City	28 / 8 (28.6)	4.1-9.8	ELISA	3.2	Amaral et al., 2006
Corn flour, cornflakes			22 / 4 (18.2)	4.5-6.2			
Popcorn			19 / 2 (10.5)	4.3-4.8			
Corn grits, White and yellow hominy			12 / -	n.d.			
Corn snacks			11 / -	n.d.			
Corn	2003	Apucarana and Andirá City	90 / 8 (8.9) 60 / 15 (16.7)	5-54 10-56.0	TLC	4	Moreno et al., 2009
Corn	2005-2006	Paraná State	74 / 12 (16)	n.d.- 3.0	LC-MS/MS	0.8	Souza et al., 2013
Corn	2010	Central-eastern Southwestern	96 / 2 (2.1%) 60 / -	N.A. n.d.	LC-MS/MS	0.001	Schmidt et al., 2015
AFM_1							
Raw milk	2001-2002	12 municipal district/ Northern	42 / 10 (24)	0.29-1.97	ELISA	0.5	Sassahara et al., 2005
Raw milk	2010	Medianeira and Serranópolis do Iguaçu	6 / 1 (16.7)	n.d. – 0.25	Fluorometer	0.10	Becker et al., 2010
Pasteurized milk			5 / -	n.d.			
UHT milk			5 / -	n.d.			
Pasteurized milk	2010-2011	11 cities	82 / -	n.d.	ELISA	0.05	Santos et al., 2014
UHT milk	2011-2012	Markets of Maringá City	152 / 133 (87.5)	0.0018 -0.12	HPLC	$1.5x\ 10^{-5}$	Silva et al., 2015

N.A.: not available; N.D.: not detected
UHT: Ultra high temperature; TLC: Thin Layer Chromatography; ELISA: Enzyme Linked Immunosorbent Assay; LC-MS/MS: Liquid Chromatography/Tandem Mass Spectrometry.

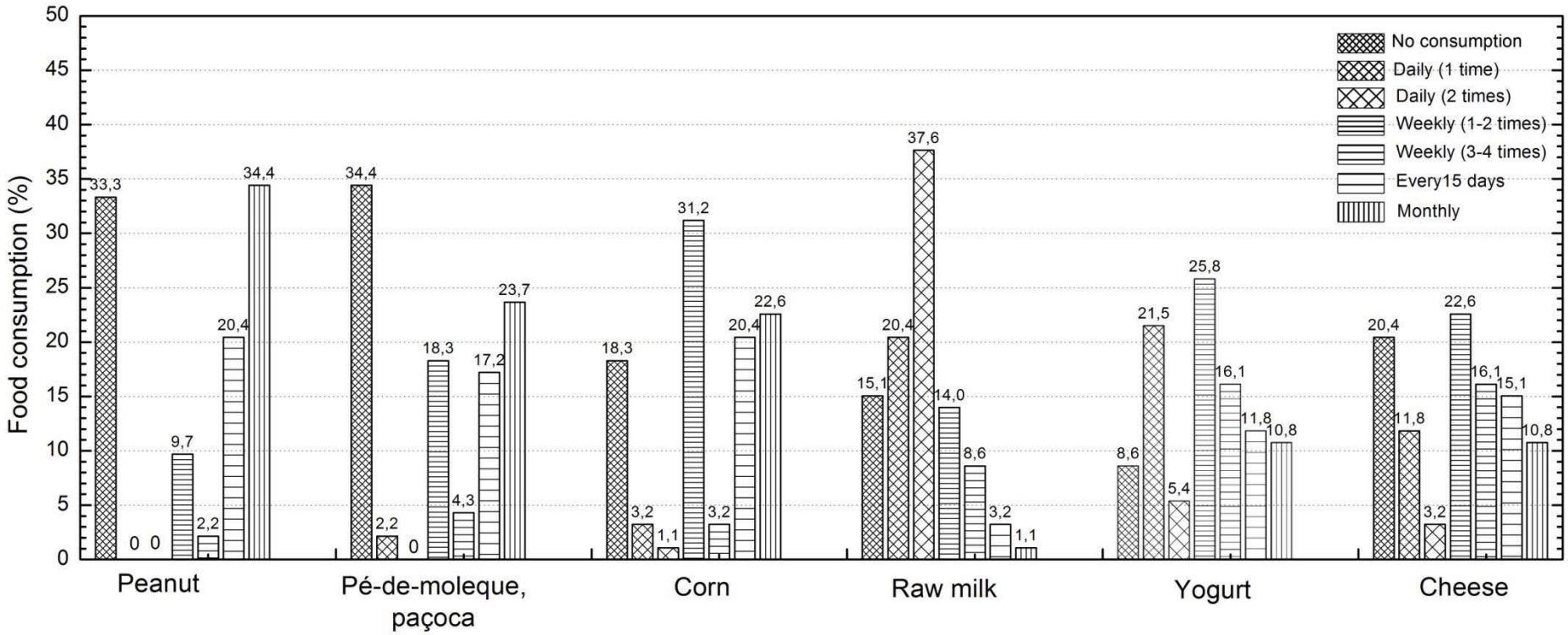

Figure 1. Frequency consumption of foods commonly contaminated with AFB_1 and AFM_1 by lactating mothers

Manuscrito 2:

Exposure assessment of infants to aflatoxin M_1 through consumption of naturally contaminated milk

Formatado nas normas do periódico Maternal and Child Nutrition

6 MANUSCRITO 2: EXPOSURE ASSESSMENT OF INFANTS TO AFLATOXIN M_1 THROUGH CONSUMPTION OF NATURALLY CONTAMINATED MILK

Running title: Aflatoxin M_1 in milk intended for infants

6.1 ABSTRACT

The aim of this study was to evaluate the exposure degree of infants to aflatoxin M_1 (AFM_1) through consumption of naturally contaminated milk. For this purpose, estimated daily intake for infants was calculated based on the AFM_1 levels analyzed in 94 breast milk (BM) samples collected in Southern Brazil, and 16 infant powdered milk (IPM) samples of national distribution. AFM_1 was detected in 5.3% (n = 94) and 43.8% (n = 16) of BM and IPM samples, with mean levels of 0.003 ng/g and 0.011 ng/g, respectively. All the IPM samples showed AFM_1 levels lower than those established by Brazilian guidelines (5 ng/g), and most of the samples (81.25%) levels were below the maximum limit by the European Commission (0.025 ng/g). The Estimated Daily Intake of AFM_1 for infants aged 0 to 12 months-old showed values from 0.019 to 0.079 ng/ kg body weight/day for BM, and 0.084 to 0.347 ng/ kg body weight/day for IPM. In conclusion, the exposure of infants to AFM_1 was low, however continuous monitoring of mycotoxin levels is essential to minimize infant health risk.

Keywords: Exposure, mycotoxin, milk, infant, safety, carry-over

6.2 Introduction

Human breast milk (BM) is recommended for the first six months of life of infants and continued breastfeeding up to two years. Breastfeeding promotes the mother-child relationship and ensures better growth and development of the newborn, providing nutrients, antibodies and leukocytes (Brasil, 2010). However, many infants and children do not receive optimal feed, wherein only 38% of infants aged 0 to 6 months worldwide and 41% of Brazilian infants of the same age are exclusively breastfed (Brasil, 2009; Brasil, 2010; WHO, 2014).

There is a special concern for infants about some trace toxins, such as aflatoxin M_1 (AFM_1) which is a monohydroxylated derivative of aflatoxin B_1 (AFB_1), present in milk. Aflatoxins (AFs) are fungal secondary metabolites that are primarily produced by *Aspergillus flavus* and *A. parasiticus*. Although, the carcinogenicity of AFM_1 is about ten fold lesser than AFB_1, the carcinogenicity of AFM_1 has been evidenced in experimental animals. AFM_1 is classified in Group 2B, possibly carcinogenic to humans (IARC, 2002). It has been suggested that children are more susceptible than adults to acute hepatotoxicity resulting from ingestion of aflatoxin and effects of toxicants due to higher metabolic rate, lower body weight, immature metabolic pathways, and incomplete development of tissues and organs (IARC, 2002; WHO, 2006a; Magoha et al., 2014). Children exposed to AFM_1 may be underweight and stunted during infancy and thus become more prone to infectious diseases (IARC, 2002; Gong et al., 2003).

AFM_1 can be used to evaluate aflatoxin exposure through diet for both human and animals. Human exposure to AFs occurs through the intake of contaminated agricultural products or through the consumption of products from animals that were fed with contaminated feed. This contamination may occur by fungal growth during harvest or improper storage (Flores-Flores et al., 2015). Because the Brazilian milk market is

characterized by big companies purchasing raw milk from different suppliers (Santili et al., 2015), companies should use high quality feedstock. Food contaminated with AFB_1 is metabolized in animals and human liver into AFM_1 by Cytochrome P450 -associated enzymes, and then distributed in serum and excreted into milk and urine. In mammals, AFM_1 can be detected in milk 12 - 24 h after the ingestion of AFB_1 (Wild and Gong, 2010; Zinedine et al., 2007). The carry-over of AFB_1 in feed metabolized into AFM_1 in milk for dairy cows was usually 1 – 2% for low-yielding cows (< 30 kg milk yield/day) and up to 6% for high-yielding cows (> 30 kg milk yield/day) (Britzi et al., 2013).

Many countries have set or proposed guidelines for maximum tolerated levels of AFM_1 in milk and milk products, because of their risk for human health. Brazilian Health Surveillance Agency (ANVISA, 2011) has not established maximum levels of AFM_1 for infant products, although the maximum limits for fluid milk and powdered milk should be 0.5 and 5 ng/g, respectively. The European guideline established the maximum levels of AFM_1 in both raw milk (0.05 ng/g) and infant formulae (0.025 ng/g; EC, 2010). Based on the potential hazard to infant (0 to 12 months-old) health due to carry-over of aflatoxin metabolites (AFM_1) into milk, the aim of this study was to evaluate the exposure of infants to AFM_1 through consumption of naturally contaminated milk.

6.3 Material and Methods

6.3.1 Breast milk and infant powdered milk samples

6.3.1.1 Breast milk samples

The healthy lactating mothers were invited to participate in this study in three hospitals in Londrina City - Brazil (Teaching Hospital of State University of Londrina, Evangelical Hospital and Municipal Maternity) from June to August 2013. The Teaching Hospital of

State University of Londrina attends patients from 250 cities of Paraná State and more than 100 cities from other States, mainly in São Paulo, Mato Grosso, Mato Grosso do Sul and Rondônia. A total of 94 lactating mothers agreed to participate in this study and the BM was collected at least 10 days after parturition. After collection, the BM was transported to laboratory in a coldbox, and it was immediately frozen at -14 °C, lyophilized and kept at -14 °C until analysis. The project was approved by Human Ethics Committee of State University of Londrina (CEP/UEL,159/2012), and all the volunteers were informed about the study protocol, a written informed-consent agreement was signed and food inquiry was applied.

The lactating mothers were instructed to complete 24-h recollection reporting all foods ingested on the day (24 h) before BM collection in order to identify potential sources of AFB_1. The foods commonly contaminated with AFB_1 and AFM_1, and consumed by lactating mothers were grouped as cereal and derivatives (rice, corn, bread, biscuit, cake, spaghetti), milk and derivatives (milk, cheese, yogurt), and others (bean, paçoca- product derived from peanuts, chocolate, nut, dried prune, ginger). The results of food consumption from each group were expressed by percentage in relation to 92 lactating mothers because two questionnaires were incompletely filled.

6.3.1.2 Infant powdered milk samples

A total of 16 samples of Brazilian IPM from different industries, belonging to three brands retailed in Brazil, were purchased in markets of Londrina City. Then they were vacuum packed and stored until analysis. The samples were recommended for infants aged 0 to 6 months (n = 7 samples), from 6 months (6) and 0 to 12 months (3). Wherein 3 samples were produced in Northeast São Paulo State, 6 in Northwest of São Paulo State, and 7 samples were imported from the United States of America, Germany and Argentina.

6.3.2 Immunoaffinity column (IAC) for clean-up of milk samples

The immunoaffinity column based on monoclonal antibody was manufactured to clean-up the milk samples. For this purpose, Hybridoma AM.3, secreting monoclonal antibody (mAb) for AFM_1 derived from SP2/0-Ag14 myeloma cell line and BALB/c spleen (Okumura et al., 1993), was cultivated in Hybridoma Serum Free Medium (HSFM, Gibco, USA) at 37 °C, 5% CO_2 (Sanyo, Japan). The IC_{50} value of Hybridoma AM.3 to AFM_1 was 6.1 pg/mL, and its cross-reactivities (%) were 0.2 (AFM_2), 0.002 (AFG_1), <0.0006 (AFG_2), 0.003 (AFB_1), <0.0006 (AFB_2). For IAC manufacture, an amount of 3 mL of Affi-gel 10 (Bio-Rad, USA) was washed with ultra-pure water at 4 °C (3-fold volume of gel) and added to 4 mg of anti-AFM_1 mAb/mL of gel, and gently mixed for 16 h at 25 °C. A solution of 1 mol/L monoethanolamine-HCl pH 8.0 was added to block the gel and mixed for 1 h at 25 °C. The gel was washed with 0.01 mol/L Phosphate Buffered Saline (PBS) pH 7.4, and an amount of 0.3 mL of coupled gel was placed in a polypropylene column (Muromac column, Japan) for AFM_1 extraction, and further analysis.

6.3.3 AFM_1 extraction from breast milk and infant powdered milk

For BM, an aliquot of 10 mL of 0.01 mol/L PBS pH 7.4 was added to 2 g of lyophilized BM and shaken at 200 rotation per minute (rpm) for 15 min. Subsequently, the volume was adjusted up to 16 mL with 0.01 mol/L PBS pH 7.4. For IPM, a volume of 20 mL of 0.01 mol/L PBS pH 7.4 was added to 4 g of IPM and 1.8 g of NaCl and shaken at 200 rpm for 15 min, and the volume was adjusted up to 25 mL with 0.01 mol/L PBS pH 7.4. After the BM and IPM samples were centrifuged at 1670 × *g* for 20 min, the fat layer was removed, and the supernatant was filtered using two glass fiber filters (GA-200 followed by GA-55, Advantec, Japan). The AFM_1 extraction by IAC was carried out with 10 mL of 0.01 mol/L

PBS pH 7.4, followed by 10 mL of reconstituted BM or IPM. The IAC was washed with 5 mL of PBS 0.01 mol/L PBS pH 7.4 and 5 mL of distilled water. The water inside the IAC was removed by adding air (30 mL). The elution was performed with 10 mL of CH_3OH:CH_3CN (1:9), and the eluate was dried in a rotary evaporator (Eyela, Japan). In this study, the rapid drying time was an important factor to maintain stability of AFM_1 for analysis.

6.3.4 Aflatoxin M_1 analysis by High Performance Liquid Chromatography (HPLC)

The dried extract was resuspended in 1 mL of mixture of ultra-pure H_2O: CH_3OH: CH_3CN (60:30:10, v/v/v). An aliquot of 10 µL was injected into HPLC equipped with an oven (CTO-10A), pump (LC20AD), degasser (DGU-20A3), auto sampler (SIL-20AHT), fluorescence detector (RF-20AXS), and reversed-phase column (XR-ODS/C8/Phenyl, 3 mm x 100 mm, 2.2 µm, Shimadzu, Japan). The excitation and emission wavelengths were 365 nm and 435 nm, respectively. The mobile phase was H_2O: CH_3OH: CH_3CN (60:30:10, v/v/v) at a flow rate of 0.5 mL/min at 50 °C, with a total run time of 10 min (Shimadzu, 2015). The AFM_1 concentration was expressed in ng of AFM_1/g.

The AFM_1 detection method was validated considering the parameters such as Limit of Detection (LOD), Limit of Quantification (LOQ), recovery test, repeatability and linearity. The LOD (signal:noise ratio, > 3:1) was 0.003 ng/g and 0.004 ng/g, and LOQ (signal:noise ratio, >10:1) was 0.016 ng/g and 0.021 for IPM and BM, respectively. The mean recovery rates of spiked BM (n = 3) at levels of 0.025, 0.05 and 0.5 ng of AFM_1/g were 110.25 ± 5.57% (RSD = 5.05), 97.21 ± 1.96% (RSD = 2.02) and 88.35 ± 1.01% (RSD = 0.01), respectively. The recovery test values for IPM at the same levels were 98.21 ± 6.43% (RSD = 6.54); 77.92 ± 1.34% (RSD = 1.72) and 71.67 ± 1.15% (RSD = 1.60). The repeatability measured by relative standard deviation was checked from replicates of

reconstituted BM and IPM samples in the recovery study. Linearity was determined from 3 calibration curves with 4 points from 0.025 to 5 ng of AFM_1/mL. The equation was y = 1090832.98 x - 10692.71, with coefficient of determination (R^2) greater than 0.99.

6.3.5 Estimated daily intake (EDI) of AFM_1 for infants

The EDI of AFM_1 for infants was estimated from relationships among the daily milk intake, *i. e*., the volume of milk consumed by infants (Bonyata, 2014), the mean AFM_1 contamination in BM and IPM samples, and average body weight for male and female (WHO, 2006b). For the volume of milk consumed by infants, it was considered that 1 g of lyophilized BM corresponded to 8 mL of reconstituted BM, and 4.52 g of IPM was required to prepare 30 mL of milk according to manufacturers. The EDI of AFM_1 was calculated according to the following formula and expressed in ng/kg of body weight/day (ng/kg b.w./day) (Santos et al., 2013).

$$\textbf{EDI}\ (\textbf{ng/kg b.w./day}) = \frac{\text{daily milk intake x mean } AFM_1 \text{ concentration}}{\text{average body weight}}$$

In this study, AFM_1 contamination in BM and IPM samples was evaluated, and values below the LOD were assumed to be 0.5 x LOD or 0 according to IPCS/GEMS (1995) criteria adopted to estimate the mycotoxin contamination when values less than the LOD were observed. These criteria considers proportion of results lesser than the LOD, firstly when all the results are over the LOD, the true means are calculated; second, when the proportion of results lesser than the LOD is lower than 60%, the mean is calculated by replacing those observations with 0.5 x LOD; third, when the proportion of results lesser than the LOD is equal or over than 60%, the mean is calculated replacing firstly those observations by 0 and

second replacing them with the LOD. In this study, the proportion of results lesser than the LOD was over than 60% for BM, and the proportion of results lesser than the LOD was lesser than 60% for IPM.

6.4 Results

6.4.1 Description of food consumption by lactating mothers

Among the foods ingested by the 92 lactating mothers during 24 h before the BM was collected, 94.5% of the mothers consumed rice, 81.5% consumed bean, and 76.1% consumed bread (Figure 1). Only a small percentage of the food ingested by participants was derived from peanut products (3.3%), nuts (2.2%), dried prune, and ginger (1.1%).

6.4.2 Breast milk and infant powdered milk analysis

Table 1 shows the AFM_1 levels in 94 BM samples. AFM_1 was detected in 5.3% (n = 94) of BM samples, with levels ranging from 0.013 to 0.025 ng AFM_1/g (mean 0.018 ng/g). Seven of the 16 IPM samples (43.8%) were contaminated with AFM_1 (LOD: 0.03 ng/g), and the levels ranged from 0.012 to 0.046 ng/g (Table 2). Three samples of IPM which are recommended for infants from 0 to 6 months of age showed contamination levels ranging from 0.015 to 0.034 ng of AFM_1/g. Furthermore, three samples for 6-month-old infants showed contamination levels from 0.012 to 0.046 ng of AFM_1/g, and one sample intended for infants aged 0 to 12 months contaminated with 0.013 ng of AFM_1/g.

6.4.3 Estimated Daily Intake (EDI) of AFM_1 for infants through breast milk and infant powdered milk consumption

The mean AFM_1 levels in BM and IPM calculated according to IPCS/GEMS (1995) criteria were 0.003 and 0.011 ng/g, respectively (Table 3). Considering the mean AFM_1 levels, EDI

values for BM ranged from 0.019 to 0.073 ng/kg b.w./day for males and 0.021 to 0.079 ng/kg b.w./day for females (Table 3). For IPM, the EDI values ranged from 0.084 to 0.324 for males and 0.091 to 0.347 ng/kg b.w./day for females.

6.5 Discussion

The human exposure to AFB_1 through diet can be estimated from aflatoxin concentration in foods and amounts that are consumed. Alternatively, the determination of aflatoxin metabolites in body fluids, such as milk and urine, is more reliable for this type of evaluation (Romero et al., 2010). In this study, the BM samples showed low AFM_1 levels (n.d.-not detectable to 0.025 ng/g), considering that samples have not been pasteurized (63°C, 30-35 min) as in BM bank, it was obtained, stored and analyzed. Similarly, in a study conducted by Iha et al. (2014), only two of 100 BM samples showed AFM_1 contamination (0.3 and 0.8 ng of AFM_1/mL). Andrade et al. (2013) analyzed 224 samples from the BM bank in the Distrito Federal, Brazil and detected no contamination with AFM_1 (LOQ = 0.005 ng/mL).

It is well known that humid and warm conditions, genetics of fungi and plants, and food storage conditions are some factors that influence mycotoxin production. Aflatoxins have been detected in a variety of agricultural commodities, but the most pronounced contamination is related to corn, peanuts, cottonseed, and tree nuts (IARC, 2002). In Brazil, traditionally the consumption of rice and bean is high, and the occurrence and levels of aflatoxins are low (Nunes et al., 2003; Silva et al., 2002). However, the occurrence of aflatoxins has been reported in corn and peanuts (Caldas et al., 2002; Romero et al., 2010; Jager et al. 2013; Jager et al., 2014). The frequency and levels of AFB_1 contamination are low in corn and derivatives from Paraná State (Souza et al., 2013; Schmidt et al., 2015). In this study, the AFM_1 detected at low levels in BM samples, was probably due to the

contamination level of AFB_1 and AFM_1 in the food consumed from local origin (Figure 1) by mothers before the BM was collected.

Polychronaky et al. (2006) reported that the consumption of grains, milk and dairy products, meat, fish, vegetables, cotton seed oil, dried fruits and peanuts by lactating mothers did not show any significant associations with AFM_1 in BM. In Italy, four out of 86 samples of BM were contaminated with AFM_1, and no relation to food consumption was found. However, the lactating mother whose milk showed high AFM_1 levels (0.14 ng/mL) had consumed a large amount of corn meal-based foods in substitution of cereal-based food (Galvano et al., 2008). In other regions of the world, such as Africa, a correlation between ingested food and AFB_1 metabolites in body fluids has been observed (Adejumo et al., 2013; El-Tras et al., 2011). This correlation could be greater in cases of acute aflatoxicosis arising from high AFB_1 levels intake by Asian and African populations, whose common diet is based on cereals and grains (FAO, 2014).

The BM has many benefits for the infant as it protects against gastrointestinal infections, reduces newborn mortality, promotes healthy growth and development; it also contains a proper balance of fats, carbohydrates, vitamins and proteins. Despite of the advantages of BM, in some situations mothers cannot breastfeed; thus, powdered milk is an alternative for providing the required nutrients to newborns and infants (Brasil, 2009; WHO, 2014). Among the 16 IPM samples analyzed in this study, seven samples were contaminated with AFM_1 (0.012 to 0.046 ng of AFM_1/g), and three of them exceeded the maximum level allowed, which is 0.025 ng of AFM_1/g (EC, 2010). However, in Brazil, there is no legislation for AFM_1 in IPM. Moreover, the Brazilian current regulation is less stringent than the European Legislation for powdered milk, and therefore, the three aforementioned samples were within the allowed limits, i.e., 5 ng of AFM_1/g (ANVISA, 2011). Two samples of IPM contaminated with AFM_1 were produced in Argentina, while five samples were produced in

Northwest of São Paulo State (Table 2) that received raw milk from local producers, and from Minas Gerais and Rio Grande do Sul States, Brazil (southeastern and southern region, respectively). AFM_1 levels in raw milk samples from different dairy farms of Brazil ranged from 0.012 to 0.725 μg/kg (Picinin et al. 2013; Santili et al., 2015).

In a study conducted by Oliveira et al. (2008), the feed intended to animals was contaminated with AFB_1 in dairy farms located in São Paulo State. The feed ingredients used were conventional corn and soybean meal prepared and locally stored, besides sugar cane (Oliveira et al., 2008). The Southeastern region of Brazil is responsible for 80% of the cultivated peanut area, especially in São Paulo State, wherein the most part of production is intercropped with sugar cane culture (Scarpin et al., 2013). Atayde et. al (2012) analyzed soil samples of four regions (West-Northwest, Midwest, West and North) of São Paulo State producing peanuts, revealing the presence of *Aspergillus* (18%). Nakai et al. (2008) isolated *A. flavus* from stored peanut samples from São Paulo State, and 93.8% of the *A. flavus* strains were producers of AFB_1 and AFB_2.

Because the AFM_1 is thermostable (120 °C) and is not readily destroyed or removed by chemical and physical treatments, monitoring AFB_1 levels in animal feeds is essential to minimize AFM_1 contamination in IPM (JECFA, 2001). It is well known that different microclimates influence the food contamination with AFs, since the major etiological determinants of fungal growth and mycotoxin production are water activity and temperature. It has been reported that the optimal temperature for AFs production were consistently at temperatures ranged from 25 to 30°C and water activity greater than 0.90, depending on the strain and substrate types (Mousa et al., 2013). During autumn and winter the availability of pasture forage in Brazil is lower, thus the animals are fed with greater amounts of feed which could be contaminated with AFB_1. Thus, a maximum level of 50 ng of AFs (AFB_1 + AFB_2 + AFG_1 +AFG_2)/g in the raw materials has been allowed for animal feed in Brazil (Brasil,

1988); and the maximum content allowed of AFB_1 for complete feeds has been established at 0.005 ng of AFB_1/g for dairy animals as cattle, sheep and goat by European Commission (EC, 2003).

The risk assessment (hazard identification, hazard characterization, exposure assessment and risk characterization) is the scientific evaluation to estimate human exposure to xenobiotic compounds through the consumption of food and provide a link between possible hazards in the food chain and the risks reflected to human health (FAO, 2004; Tsakiris et al., 2013). Although there are differences in the frequency of feed and variations in the volume of milk consumed by infants, it is possible to estimate the exposure of infants to AFM_1 using the EDI. It is noteworthy that male and female infants from 0 to 12 months of age show differences in body weight and milk consumption; thus, the EDI level is affected by these factors. The EDI values of AFM_1 for infants decreased as the age is increased, showing the susceptibility of younger infants (Table 3).

In this study, EDI values (0.019 - 0.347 ng of AFM_1/kg b.w./day) were lower than those reported by other authors in Brazil. Oliveira et al. (1997) reported an EDI of 3.7 ng of AFM_1/kg b.w./day for a 4-month-old infant weighing 6 kg, representing a daily intake of 22 ng of AFM_1. In another study, the EDI ranged from 0.106 to 1.04 ng of AFM_1/kg b.w./day for children (23 kg) who consumed 400 mL of powdered milk with 0.006 to 0.061 ng of AFM_1/g, respectively (Shundo et al., 2009; Santili et al., 2015). There are few data available on the AFM_1 intake by infants (0 to 12 months-old) in the Brazilian literature, and around the world the most of EDI data were about children (1 to 8 years-old). EDI values of different countries (Spain, Argentina and Thailand) ranged from 0.16 to 3.70 ng of AFM_1/kg b.w./day (Cano-Sancho et al., 2010; Alonso et al., 2010; Ruangwises et al., 2011). Recently, Ismail et al. (2016) reported EDI values for male and female children from Paquistan ranging from 1.68 to 5.45 ng of AFM_1/kg b.w./day, and the highest EDI value (5.45 ng of AFM_1/kg

b.w./day) was 15.7 fold higher than obtained in this study (Table 3). According to The Joint FAO/WHO Expert Committee on Food Additives (JECFA), the average dietary intake of AFM_1 for adults is 3.5 ng/person/day based on the Latin American diet (JECFA, 2001).

Based on studies in male Fischer rat, Kuiper-Goodman (1990) has determined a No Observed Effect Level (NOEL) for AFM_1 of <2.5 µg/kg body weight/day. Furthermore, the author also have proposed the dose of 0.2 ng/kg body weight/day as a "safe dose"*, i.e.*, 50% of the animals would have developed tumors (TD_{50}) dividing by a large safety factor of 50,000. In this study, the EDI of AFM_1 for BM and IPM was much lower than the NOEL, and EDI for BM was lower than the "safe dose" indicating that BM was safe to be consumed by infants.

In some cases, the results of exposure assessment (Estimated Probable Daily Intake) are compared with the hazard characterization Tolerable Daily Intake (TDI) to indicate the degree of human exposition to mycotoxin (Kuiper-Goodman, 1995). However, this hazard assessment is not applied for carcinogenic toxins such as aflatoxin B_1, and any small dose will have a proportional effect. Therefore, the JECFA does not allocate a Provisional Tolerable Daily Intake (PTDI) or Provisional Tolerable Weekly Intake (PTWI). Alternatively, the level of contaminant in food should be reduced so as to be As Low as Reasonably Achievable (ALARA). The ALARA level is the concentration of a substance that cannot be eliminated from a food without involving the discard altogether or without severely compromising of major food supplies (FAO, 2004).

Once the AFs are absorbed by the gastrointestinal and respiratory tract, they reach the blood circulation and are distributed into several tissues, primarily the liver. AFB_1 can be detoxified to AFM_1 or metabolized by cytochrome P450 to aflatoxin-8,9-epoxide, a reactive form that binds to DNA and albumin from blood, forming adducts and causing DNA damage (IARC, 2002). Carcinogenic and acute toxicity responses to AFB_1 are dependent on

metabolic activation. However, in contrast to AFB_1, it has been demonstrated a direct cytotoxic effect of AFM_1 on human cells that does not require prior metabolic activation (Neal et al., 1998). Early childhood exposure to aflatoxins is difficult to be detected by clinical signals, but it may be critical determinants for immediate and later health effects. Therefore, continuous monitoring of aflatoxin occurrence in foods, particularly in IPM, is necessary because this food is an important alternative for infants' food nutrition in situations where mothers cannot breastfeed. Nevertheless, breastfeeding is important and must be encouraged for children up to two years of age when possible.

In this study, exposure of infants to AFM_1 was low indicating that lactating mothers and dairy cows had low exposure to AFB_1. Despite the low AFM_1 levels in the BM and IPM samples, continuous monitoring of AFM_1 and establishment of maximum AFM_1 limit by Brazilian legislation for infant formulas are required due to the higher susceptibility of infants to mycotoxin compared with adults.

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Table 1. Distribution of aflatoxin M_1 levels in naturally contaminated breast milk samples from lactating mothers of Paraná State, Brazil

Breast milk	**Aflatoxin M_1**		
n	**Positive samples (%)**	**Range (ng/g)**	**Mean[a] (ng/g)**
89	-	n.d.	-
3	3.2	0.013 – 0.015	0.014
2	2.1	0.022 – 0.025	0.024
Total: 94	**5.3**	**n.d. – 0.025**	**0.018**

The distribution of aflatoxin M_1 levels was divided in: a) < LOD, b) between LOD and LOQ, and c) >LOQ
Limit of detection (LOD): 0.004 ng/g; Limit of quantification (LOQ): 0.021 ng/g
[a] Mean of positive samples
n: number of samples; n.d.: not detectable

Table 2. Aflatoxin M_1 levels in infant powdered milk retailed in Brazil

Age	*n* total	*n*	Product origin	Range (ng/g)	Mean[c] (ng/g)	Positive samples (%)
0-6 months	7	2	Northeast of São Paulo State	n.d.	0.022	42.9
		3	Northwest of São Paulo State	n.d. - 0.016		
		2	Buenos Aires – Argentina[a]	n.d. - 0.034		
From 6 months	6	1	Northeast of São Paulo State	n.d.	0.030	50.0
		3	Northwest of São Paulo State	0.012 - 0.046		
		2	Buenos Aires – Argentina[a]	n.d.		
0-12 months	3	1	Fussener Strasse – Germany[b]	n.d.	0.013	33.3
		1	Florham Park – USA[b]	n.d.		
		1	Buenos Aires – Argentina[a]	0.013		
Total	**16**			**n.d. – 0.046**	**0.024**	**43.8**

Limit of detection: 0.003 ng/g; Limit of quantification: 0.016 ng/g
[a] IPM produced in Argentina and imported by São Paulo – São Paulo State
[b] IPM imported by São Bernardo do Campo City – São Paulo State
[c] Mean of positive samples
n: number of samples
n.d.: not detectable

Table 3. Age group, milk consumption, average weight and Estimated Daily Intake of AFM_1 by infants through naturally contaminated breast milk and powdered milk*

Age	Milk consumption (mL)[a]	Average weight (kg)[b]		Estimated Daily Intake (ng/kg b. w./day)			
				BM		IPM	
		M	F	M	F	M	F
1 week	483	3.3	3.2	0.055	0.057	0.243	0.250
1-6 months	880	4.5 - 7.9	4.2 - 7.3	0.073 - 0.042	0.079 - 0.045	0.324 - 0.185	0.347 - 0.200
7 months	875	8.3	7.6	0.040	0.043	0.175	0.191
12 months	487	9.6	8.9	0.019	0.021	0.084	0.091

* Considering IPCS/GEMS criteria (1995), the mean of AFM_1 contamination in BM and IPM were 0.003 and 0.011 ng/g, respectively

[a] Bonyata, 2014; [b] WHO, 2006b

M: male; F: female; b. w.; body weight

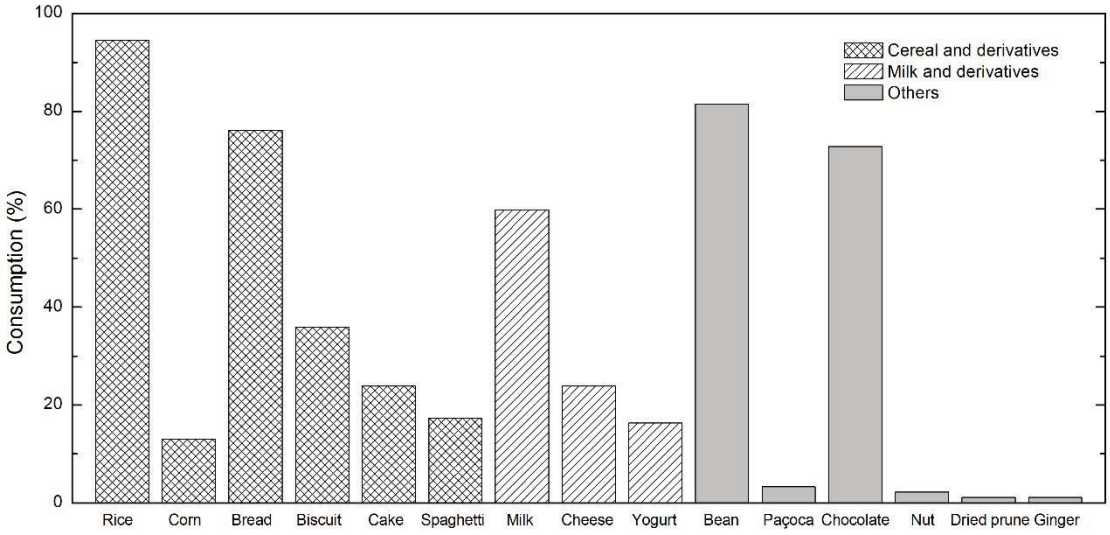

Fig 1. Food ingested by lactating mothers 24 h before breast milk collection (n = 92) was grouped as Cereal and derivatives, Milk and derivatives, and Others.

Manuscrito 3:

Effect of administration of single oral subclinical doses of aflatoxin B_1 in the liver and gut microbiota in C57Bl/6 mice

7 MANUSCRITO 3: EFFECT OF ADMINISTRATION OF SINGLE ORAL SUBCLINICAL DOSES OF AFLATOXIN B_1 IN THE LIVER AND GUT MICROBIOTA IN C57BL/6 MICE

7.1 ABSTRACT

Aflatoxin B_1 (AFB_1) is a secondary metabolite from fungi that present toxic, mutagenic, and carcinogenic effect. It is found in both food and feed resulting in human and animal risk. The purpose of the present study was to investigate the effects of single oral administration of a subclinical dose of AFB_1 in the liver and gut microbiota in mice. A total of 25 male C57Bl/6 mice were allocated in five groups. Three groups were treated with a single oral dose of AFB_1 (44 μg of AFB_1/kg of body weight, b.w., 442 μg of AFB_1/kg b. w., and 663 μg of AFB_1/kg of b. w.) on the first day and euthanized on the 5th day. Two control groups were treated only with water and vehicle (saline:ethanol, 95:5). For liver, it was included determination of cytokines (IL-4, IFN-γ, IL-17) levels and histopathology analysis was performed. Sequencing of the V4 region of the 16S rRNA was performed with an Illumina MiSeq sequencer to evaluate the gut microbiota composition. The animals treated with 663 μg of AFB_1/kg of b. w. had liver lesions such as necrosis, infiltrate inflammatory, megalocytosis, cell and nuclear vacuolation; and a significant upregulation of IL-4 and IFN-γ levels in the liver ($p < 0.05$). In addition, this group presented increased family of Lachnospiraceae on the gut microbiota. In conclusion, single oral subclinical dose of AFB_1 exposure can induce liver tissue lesions, liver cytokine modulation and possible change in gut microbiota in C57Bl/6 mice.

Keywords: cytokines, gut microbiota, histopathology, inflammation, mycotoxin

7.2 INTRODUCTION

Mycotoxins are naturally occurring toxic metabolites produced by fungi, with emphasis in aflatoxins (AFs). It is widely assumed that AFs can be produced on a wide range of agricultural commodities and under a diverse range of conditions worldwide, including crops, processed food and feed (FAO, 2016). The AF contamination represents a potential risk factor to both human and animal health as they are responsible for many different toxicities (Pleadin et al., 2014). It was estimated that 25% of the crop harvest in the world are contaminated with mycotoxin, and approximately 5 billion people in developing countries are exposed to amounts of AFs (Willians et al., 2004; Fetaih et al., 2014).

Four major structurally related AFs have been identified which were designated as B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2). However, AFB_1 is considered as the most potent hepatocarcinogen, it has been classified carcinogenic to human (Group 1) by the International Agency for Research on Cancer (IARC, 2012; Diao et al., 2013). Acute exposure to high concentrations of AFs in humans can cause hemorrhage, vomiting, acute liver damage, pulmonary edema, convulsions, coma, and death. Clinical signs of aflatoxicosis in animals include gastrointestinal dysfunction, reduced reproductivity, impaired food conversion, slower rates of growth, anemia, and jaundice (Diaz, Murcia, 2011; Bbosa et al., 2013).

The mammalian liver is the major drug-detoxifying organ, responsible for metabolic activation, elimination of toxic chemicals and metabolic intermediates, including AFs (Liska, 1998). Absorption of AFs in the rat small intestine is a rapid process, absorbed AFB_1 reaches the liver through the portal system, and it is metabolized by cytochrome P450 (CYP). AFB_1 is metabolized to the active form to exert carcinogenic activity, or converted to less toxic form. AFB_1 metabolites are transferred from the hepatic cells to the blood and

bile, and then excreted in the urine and feces, respectively (Kumagai et al., 1989; Kensler, 2003; EFSA, 2007).

On the other hand, the role of extra-hepatic metabolism of aflatoxin, particularly in the small intestine, may be important in modulating the toxic and carcinogenic effects *in vivo*. Enterocytes of the small intestinal epithelium contain high levels of CYP 3A enzymes and can activate aflatoxin, possibly limiting the systemic absorption of AFB_1 (Wild, Turner, 2002). Furthermore, it was experimentally demonstrated that rats injected intravenously with AFB_1 excreted a significant amount of AFB_1 and its metabolites into the intestinal lumen, not via bile, as another route of aflatoxin excretion (Kumagai et al., 1989). Consequently, the contact of AFs and its metabolites in the intestine and feces could affect the gut microbiota composition.

Recently, studies are focused on microbial composition in human and animal intestine (Mowat, Agace, 2014). Researchers have shown that xenobiotic compound exposure modify the composition of microbial communities, and it was associated with disease because the gut homeostasis is disrupted (Wang et al., 2015). Among the main contributions of the microbiota to the host were included development of gut-associated lymphoid tissues (GALTs) and the polarization of gut-immune responses (Kamada et al., 2013; Mowat, Agace, 2014). Mycotoxins are able to compromise several key functions of the gastrointestinal tract. Nevertheless, the effect of mycotoxin on the gut microbiota composition is largely unknown (Grenier, Applegate, 2013).

In vivo experiments, mice have been exposed to acute or chronic AFB_1 dose for an extend period to find different effects and impact of AFB_1 exposure on animals (Ilic et al., 2010; Abdulrazzaq et al., 2011; Jha et al., 2013; Mulder et al., 2015). The present study investigated the effects of single oral subclinical dose of AFB_1 on hepatic tissue, and gut microbiota in C57Bl/6 mice.

7.3 MATERIAL AND METHODS

7.3.1 Aflatoxin B_1 standard

The AFB_1 standard from *Aspergillus flavus* (A663, Sigma, USA) was quantified according to Instituto Adolfo Lutz (IAL, 2008). The molar absorptivity of AFB_1 considered for calculating AFB_1 concentration in methanol was 21,800 at 360 nm.

7.3.2 Aflatoxin B_1 dose and exposure time criteria

The lowest AFB_1 dose (44 μg/kg of b.w.) was calculated based on maximum levels allowed in both rice and bean which is 5 μg/kg (EC, 2010; ANVISA, 2011) and it was considered average consumption of bean (183 g/day) and rice (160 g/day) as traditional diet in Brazil (IBGE, 2011). The highest dose of AFB_1 (663 μg/kg of b. w.) was based on chronic AFB_1 dose used in other studies (Jha et al., 2013; Feitah et al., 2014). The time length of aflatoxin exposure was choosen to evaluate how single dose of AFB_1 can affect gut microbiota, since there are no studies evaluating this effect in the literature.

7.3.3 Animals, housing and experimental design

A total of 25 male C57Bl/6 mice (10 weeks-old, average weight: 22.55 ± 0.89 g) were obtained from University of São Paulo – Ribeirão Preto City, Brazil. The C57Bl/6 mice were chosen for this study because is the most susceptible strain to the acute effect of aflatoxin B_1 (Almeida et al., 1996). The mice were acclimatized for three weeks and housed in polyethylene boxes on a bedding of wood shavings, maintained under standard conditions of temperature about 25 °C with regular 12h light/12h dark cycle. All mice were given standard rodent pelleted food and *ad libitum* water.

The experimental design used in this study was randomized with five repetitions (each animal represented one repetition) for each group. Group 1 was untreated control mice;

Group 2 received only the vehicle (saline:ethanol, 95:5) on the first day; Group 3, received a single dose of 44 µg of AFB_1/kg of b. w. on the first day; Group 4 received a single dose of 442 µg of AFB_1/kg of b. w.; and Group 5 received a single dose of 663 µg of AFB_1/kg of b. w. The AFB_1 was administered by oral gavage (0.1 mL each 10 g of body weight), wherein AFB_1 was suspended in saline:ethanol (95:5). During 5 days of experiment period, the behavior and feces of animals was observed. After 5 days the animals were weighed, euthanized and liver removed. Liver was collected, weighed and divided in two subsamples, one portion was set aside for histopathology analysis and the other portion of liver cells was stored at - 80°C for cytokine determination. In addition, mice feces from large intestine content were stored at - 80°C for later evaluation of the fecal intestinal microbiota population. This study was approved by Committee of animal ethics of State University of Londrina (n° 26362.2014.65).

7.3.4 Cytokine Assays

The liver samples was macerated in adjusted volume of 0.1M Phosphate Buffer Saline (PBS) pH 7.4 (1:5000 dilution). Posteriorly, IL-4, IFN-γ and IL-17 levels in the liver were determined by commercial sandwich ELISA kits from BioSource International, Inc (Camarillo, California, USA), according to the manufacturer's instructions. The reaction was revealed with avidin-peroxidase followed by the substrate mixture containing hydrogen peroxide and 3, 3', 5, 5'-tetramethylbenzidine (TMB) as a chromogen. The absorbance was read into an ELISA microplate reader (iMarkTM, Bio-Rad, USA) at 450 nm. The absolute cytokine level was calculated based on a standard curve provided by the manufacturer (IL-4 and IL-17, sensitivity: 4 pg/mL, standard curve range: 4-500 pg/mL; IFN- γ, 15 pg/mL, 15-2000 pg/mL).

7.3.5 Histopathological analysis

The liver was dissected from the animals and fixed in 10% paraformaldehyde solution at room temperature for 72h. It was dehydrated in ascending grades of ethyl alcohol (75, 95 and 100%), cleared, and embedded in paraffin by routine techniques. For each animal, two tissue sections of 5 μm were stained with hematoxylin-eosin (HE) for histopathological evaluation. The lesion score was calculated taking into account the degree of severity (severity factor, from one to three) and the extend of each lesion (according to intensity or observed frequency, scored from zero to three) (Table 1). The extend of each lesion was scored as follows, megalocytosis (the mean of 5 field per histological section): 1-10 = 0, 11-20 = 1+, 21-30 = 2+, 31-40 = 3+; inflammatory infiltrate and nuclear vacuolation (histological section): absent = 0, 1 = 1+, >2 = 2+; hepatic cell vacuolation (histological section): mild = 0, moderate = 1+; and necrosis (histological section): absent = 0, mild = 1+, moderate = 2+, severe = 3+. For each type of lesion, the score of the extend was multiplied by the severity factor, and final score (sum of all type of lesion) in each tissue ranged from zero to 20 for liver, with maximal score was 18 for each animal group (Gerez et al., 2015).

7.3.6 Bacterial DNA extraction from mice feces, 16S rRNA gene PCR and sequencing

The fecal samples from large intestinal content of vehicle and 663 μg/ kg of b. w. group were prepared and sequenced according to Costa et al. (2015a). The DNA extraction was performed using the E.Z.N.A.® Stool DNA Kit (Omega Bio-Tek, USA) following the manufacture instruction for Stool DNA protocol for pathogen detection. Then, PCR (Polymerase Chain Reaction) amplification of the V4 region of the 16S rRNA gene was performed using the primers forward S-D-Bact-0564-a-S-15 (5'-AYTGGGYDTAAAGNG-3') and reverse S-D-Bact-0785-bA-18 (5'-TACNVGGGTATCTAATCC-3') (Klindworth et al., 2013). PCR was carried out in two steps: first, 2.5 μL of DNA template were added to a

mixture containing 9 µL of water, 12.5 µL of Kapa 2X ReadMix (Kapa Biosystems. USA) and 0.5 µL of each 16S primer (10 pmol/µL). The mixture was subjected to the following PCR conditions: 3 min at 94°C for denaturing, and 26 cycles of 45s at 94°C for denaturing, 60s at 53°C for annealing and 90s at 72°C for elongation followed by a final period of 10 min at 72°C and kept at 4°C. PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter). The second PCR was carried out by adding 4 µL of the purified product to a mixture containing 9.6 µL of water, 20 µL of 2X Ready Mix and 3.2 µL of each Illumina index primer (2.5pmol/µL) containing an overlapping region to the 16S rRNA primers. PCR conditions consisted of 3 min at 94°C, and 7 cycles of 45s at 94°C, 60s at 50°C and 90s at 72°C and a final period of 10 min at 72°C and kept at 4°C. A second purification with magnetic beads was performed. Samples were sequenced with an Illumina MiSeq with 250 cycles from each end (500bp) at the University of Guelph Genomics Facility.

7.3.7 Statistical analysis

Data were analyzed by using Statistica 10.0 (Stat Soft Inc.) and presented as mean ± standard deviation for cytokines level and liver histophatology. Before the analysis, homogeneity of variances (Levene´s Test) and data normal distribution (Shapiro-Wilk's Test) were done. The one-way analysis of variance (ANOVA) followed by the Tukey's Test were performed, and p value < 0.05 was considered as significant.

The bacterial community analysis was performed with the software Mothur (v1.36) (Schloss et al., 2009). Sequences were classified at the genus level using the Ribossonal DataBase Project (RDP) databank (Cole et al., 2005). Good's coverage was calculated in order to ensure representative sub-samples. Rarefaction curves plotting the number of genera by the number of reads were calculated to demonstrate the richness (number of species in

the community) within each sample. Diversity (estimate richness and its distribution) was estimated by the inverse Simpson's index and relative abundances compared from the main phyla and genus (abundance >1%) were compared between groups using a non-parametric test (Mann-Whitney test) with significance level of 5%. The dissimilarity of bacterial composition (the different species present in each sample) was measured by the classic Jaccard index and the community structure (the different species and how are distributed in each sample) was evaluated by the Yue and Clayton method. The similarity between the samples was then, visualized by dendograms using the Figtree software (website) and the Principal Coordinate Analysis (PCoA). The parsimony test and the analysis of molecular variance (AMOVA) were used to compare the similarity of community membership and structure between the two groups. The indicator analysis was used in order to identify genera that were representative of each group.

7.4 RESULTS

7.4.1 Absolute body and organs weight of mice after aflatoxin B_1 exposure

Single oral administration of AFB_1 in mice did not decrease the gain of body weight or alteration in liver weight in animals treated with AFB_1 in relation to control untreated group ($p > 0.05$, data not shown). Diarrhea, presence of blood in the feces or behavioral alteration were not detected signs, therefore the dose used in this experiment was considered as subclinical.

7.4.2 Aflatoxin B_1 on expression of cytokines in the liver

The expression of three cytokines (IL-4, IFN-γ, IL-17) was performed in the liver (Figure 1). A significant upregulation of IL-4 and IFN-γ were observed in mice treated with 663 μg of AFB_1/kg of b. w. in comparison to vehicle group ($p < 0.05$), but not for IL-17 cytokines

levels ($p > 0.05$) (Figure 1). However, there was difference among animals treated with 44 and 442 µg of AFB_1/kg of b. w. compared to animals treated with 663 µg of AFB_1/kg of b. w ($p > 0.05$).

7.4.3 Evaluation of aflatoxin B_1 on histological lesions in the liver tissue

The main lesions evaluated in liver were hepatic cell vacuolation, megalocytosis, nuclear vacuolation, inflammatory infiltrate and necrosis. Lesions recorded in liver were considered moderate to severe for mice treated with AFB_1. As exemplified in Figure 2, overall microscopic alterations resulted in a higher score in group treated with 663 µg of AFB_1/ kg of b. w (lesion score of 12.40 ± 3.29) when compared to vehicle group (3.80 ± 2.77) ($p < 0.05$). Animals treated with 663 µg of AFB_1/kg of b. w. showed more pronounced intensity of megalocytosis, nuclear vacuolation and necrosis in comparison to the control group, whereas the main lesion for animal treated with 44 and 442 µg of AFB_1/kg of b. w. was necrosis (5.33 ± 1.51 and 7.0 ± 3.67, respectively).

7.4.4 Aflatoxin B_1 effect on the gut microbial community

A total of 693,477 good quality reads were obtained and a subsample ($n = 29,157$ reads) was used to avoid bias caused by non-uniform samples sizes, which achieved good coverage (medium of 99.9%). Rarefaction curves which represents richness are presented in Figure 3 and the relative abundances at the phylum and genus levels found in each group are displayed in Figure 4. Sequences were classified into seven different phyla, and the most abundant bacteria were Firmicutes (59.64% and 60.92% for Vehicle and AFB_1 663 µg/kg of b. w. group, respectively) followed by Verrucomicrobiota (15.30 % and 15.80%) and Proteobacteria (2.50% and 7.68%) (Figure 4A). At the genus level, a total of 15 genera were found, of which 8 were I*ncertae sedis.* The predominant bacteria belonged to the

Lachnospiraceae family (17.95% and 27.59%), followed by *Akkermansia* sp. (15.26% and 15.79%) and *Lactobacillus* sp. (17.61% and 9.38%) (Figure 4B). There were significantly less *Mucispirillum* from Firmicutes in animals treated with 663 μg of AFB_1/kg of b. w. In contrast, the treatment with AFB_1 resulted in significantly more Lachnospiraceae ($p < 0.05$).

A total of three genera were representatives of each analyzed group, the indicator bacteria of the vehicle group belonged to the Staphylococcaceae (unclassified and *Staphylococcus* sp.) and Ruminococcaceae (*Pseudoflavonifractor* sp.) families, while animals treated with 663 μg of AFB_1/kg of b. w. had Rikenellaceae (*Alistipes* sp.), Bacillales_Incertae_Sedis_XI (*Gemella* sp.) and Comamonadaceae (*Delftia* sp.) families as good indicators for that group.

Figure 5 shows the graphical representation of the principal coordinate analysis (PCoA) obtained with the Classic Jaccard (Figure 5A) and the Yue and Clayton (Figure 5B) analysis. Despite the tendency to form two distinct clusters (Figure 6), especially regarding community membership (Jaccard, Figure 6A), the statistical analysis revealed that single subclinical dose of AFB_1 had no impact on the intestinal microbiota of those animals by Parsimony and AMOVA (data not shown, $p > 0.05$).

7.5 DISCUSSION

The cytokines are important mediators in the immune response and animals treated with 663 μg of AFB_1/kg of b. w. showed upregulation of hepatic IL-4 and IFN-γ levels, an anti-inflammatory and inflammatory cytokine, respectively. According to Schroder et al., (2004), INF-γ orchestrates leukocyte attraction and directs growth, maturation, and differentiation of many cell types and negative regulators of INF-γ production including IL-4.

There is no consensus on cytokine response by AFs exposure. The increase of pro-inflammatory cytokine, such as INF-γ and TNFα, in different organs was reported by other authors (Chaytor et al., 2011; Greiner et al., 2013; Li et al., 2014; Monson et al., 2015). Marin et al. (2002) and Meissonnier et al. (2008) showed an increase in the anti-inflammatory cytokine IL-10 in spleen cells. Qian et al. (2014) demonstrated difference in the expression of cytokines by lymphocytes from rat spleen after short-term exposure (1-week) and after 5-weeks of AFB_1 exposure (5- 75 μg/kg of b.w.). It was detected decreased expression of IL-4 and IFN-γ by lymphocytes in both groups, but, after 5-weeks detected elevated IFN-γ expression by CD4+ T cells and increased expression of TNF-α by $CD3^{-}CD8a^{+}$ NK cells, inflammatory cytokines. The difference obtained in the present study, may be due time to a single higher dose and different organ and species.

As this study was earlier (5 days), thus prior to the adaptive response, increases in IL-4 and INF-γ should have been through innate immune cell activation. As expected there was no change in IL-17 level in animals treated with AFB_1 compared to vehicle group, a cytokine produced by Th17 cell, belonging to the adaptive response. However, there was difference among animals treated with 44 and 442 μg of AFB_1/kg of b. w. compared to animals treated with 663 μg of AFB_1/kg of b. w. The impact on mixed response of IL-4 and INF-γ in Th2 or Th1 response polarization or involvement in hepatic injury requires further studies.

In addition, immune-mediated mechanism can be either an independent or an interactive factor to trigger the pathogenesis of liver injury (Wang et al., 2014). The liver plays a central role in preventing accumulation of a range of compounds. The metabolism results in increased polarity of the compound converting them into a form suitable for excretion. As the process of xenobiotic metabolism requires several biochemical transformations, and some intermediates mediate toxic responses, the liver is potentially

susceptible to injury during the act of performing its function (EFSA, 2007; Ierapetritou et al., 2009). The liver has a big population of Kupffer cells, dendritic cells and Natural Killer (NK) cells. It has been demonstrated that hepatic NK cells produce large amounts of IFN-γ upon activation, modulating T cell responses in the liver, promoting intracellular changes in endothelial cells and hepatocytes. Besides that, it can directly promote necroptosis of hepatocytes or cell lysis (Baeck, Tacke, 2014; Wang et al., 2014). In this study, deleterious effects in the liver were observed with single oral subclinical dose of AFB_1. Hepatic cellular architecture and organization changes in treated groups were observed by histopathological examination. Necrotic lesions were observed in all animals groups treated with AFB_1 (44, 442 and 663 µg/kg of b. w.). The histological alterations as megalocytosis, nuclear vacuolation and inflammatory infiltrate were pronounced only in animals treated with 663 µg of AFB_1/kg of b. w. (Figure 2). The same liver alterations were observed in different frequency and/or intensity by previous researches (Sharma et al, 2011; Colakoglu, Donmez, 2012). The effects of AFs on histopathological changes are directly correlated with the concentration of AFB_1 and the duration of the exposure (Baptista et al., 2008).

Besides the liver damage caused by AFB_1, it was also evaluated the possibility of AFB_1 change the gut microbial composition. In the last years, mammal-associated microbiomes have been shown to influence the host metabolism, homeostasis, and inflammation. The effects of xenobiotic compounds on the microbial community composition changes have been documented previously in some mammals (Breton et al., 2013; Guo et al., 2013; Costa et al., 2015a). In this study, single dose of AFB_1 (663 µg/kg of b. w.) had no impact on mice microbiota from large intestine content. However, the PCoA and dendograms representing community similarity among samples, especially regarding community composition (Classic Jaccard) showed a tendency for cluster formation,

suggesting that a bigger number of samples could potentially reveal a more evident effect of AFB_1 on the intestinal microbiota of mice.

The predominance of Firmicutes in fecal samples was consistent with the literature (Yan et al., 2011). However, there are some conflicting results regarding relative abundance in gut microbiota of mice, studies reported Bacteroidetes along with Firmicutes as the major dominated phyla (Hildebrand et al., 2013; Nguyen et al., 2015). In this study, Verrucomicrobia appeared as the second most prevalent phylum, followed by Proteobateria and Bacteroidetes (Figure 6A). Those differences are likely due to the different methodologies applied to evaluate different regions of the 16S rRNA gene, as well as different mouse strains and age (Hildebrand et al., 2013).

The relative abundance of the Firmicutes and Bacteroidetes phyla were not impacted by AFB_1 treatment. However, when exploring deeper taxonomic classifications, there was greater relative abundance of the Lachnospiraceae family from the Firmicutes phylum with treatment of single dose of AFB_1 (Figure 6B). In disagreement with Wang et al. (2015) that evaluated the gut microbiota of rats treated with 5, 20 and 75 μg of AFB_1/ kg of b. w. during 4 weeks, and reported the largest decrease in two Lactobacillales, *Streptococcus sp.* and *Lactococcus sp*., appearing in a dose-dependent manner.

Lachnospiraceae are included as major constituents of mammalian gastrointestinal microbiota, constituting some of the main families of active bacteria present in the distal gut (Meehan, Beiko, 2014, Costa et al., 2015b). Members of this family have been associated to obesity, as well as cancer protection in human colon, mainly due to the association of many species within the group with production of butyric acid (Bultman, Jobin, 2014; Meehan, Beiko, 2014; Vital et al., 2014). Also, it is well known as an indicator of fecal contamination in water and sewage (MecLellan et al., 2013). Reeves et al. (2012) reported that members of the bacterial family Lachnospiraceae could partially restore colonization resistance against

Clostridium difficile. Guo et al. (2014) showed effects of mycotoxin ochratoxin A (OTA) on gut microbiota of rats showed that anaerobic strains may be more sensitive than facultative anaerobic strains. However, in this study, there was an increasing of Lachnospiraceae family which is considered anaerobic.

It has been demonstrated that mycotoxins are able to compromise functions of the gastrointestinal tract, including decreased surface area for nutrients absorption, modulation of nutrient transporters and loss of barrier function. Furthermore, some mycotoxins facilitate persistence of intestinal pathogens (Greiner, Applegate, 2013). Saint-Cyr et al. (2013) hypothesized that a mycotoxin (deoxynivalenol) may promote the passage of pathogenic microorganisms present in food and water across the intestinal epithelium. Nevertheless, there are only few studies addressing the effect of mycotoxins on the gut microbiota composition, hindering the understanding of its action in the body (Saint-Cyr et al., 2013; Guo et al., 2014; Wang et al., 2015). In this study, the indicators bacteria found in each group (Vehicle and AFB_1 663 µg/kg of b. w.) also suggests changes at genus level, can be a good indicator to assess compositional changes on the gut bacterial community with AFB_1 treatment. The small sample size used in this study should be pointed as its major limitation, since it has likely limited its statistical power. However, the results presented here suggests that AFB_1 can affect the gut microbiota it has been previously shown. Further studies are necessary for a better understanding of the consequences from the bacterial changes caused by AFB_1 exposure. Additionally, the evaluation of different higher doses is necessary to be performed.

In this study, increasing subclinical doses of AFB_1 showed more pronounced toxic effects. There are many studies about chronic AFB_1 exposition in higher dose in animal models, but it is important to evaluate how low doses and frequency of AFB_1 exposition

could affect the human and animals, specially the AFB_1 effect on the gut microbiota composition.

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Table 1. Score for extend of each lesion and severity factor for different types of lesions in the liver to assess histological analysis

LIVER		
Type of lesions	**Extend of lesion**	**Severity factor**
Nuclear vacuolation	Absent = 0 1 = 1+ ≥ 2 = 2+	1
Inflammatory infiltrate	Absent = 0 1 = 1+ ≥ 2 = 2+	1
Hepatic cell vacuolation	Mild = 0 Moderate = 1+	1
Megalocytosis	1-10 = 0 11-20 = 1+ 21-30 = 2+ 31-40 = 3+	2
Necrosis	Absent = 0 Mild = 1+ Moderate = 2+ Severe = 3+	3

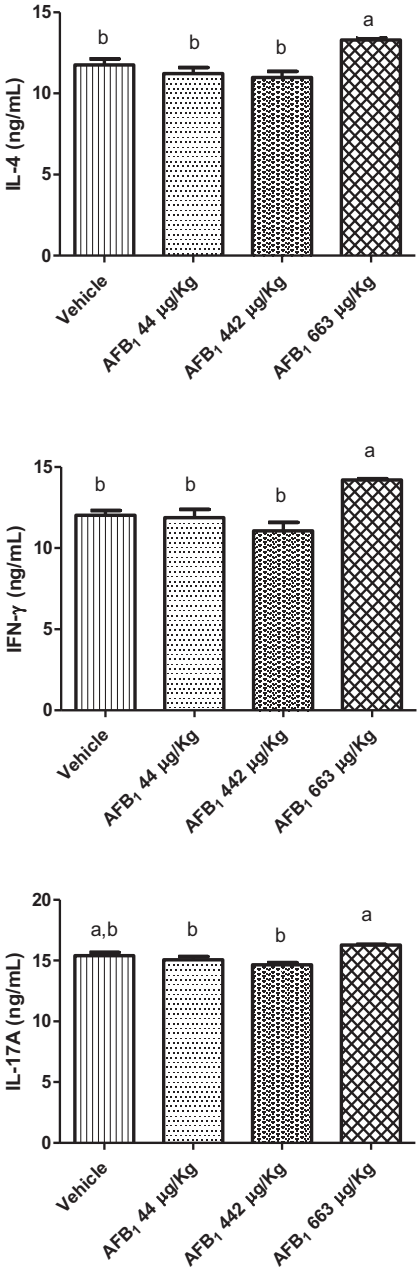

Figure 1. Effect of aflatoxin B_1 on the expression of A) IL-4, B) INF-γ and C) IL-17 in the liver after five days post-aflatoxin exposure. Data was expressed as mean ± SD, $n = 5$. Means without a common letter were statistically significant ($p < 0.05$) by the Tukey's Test.

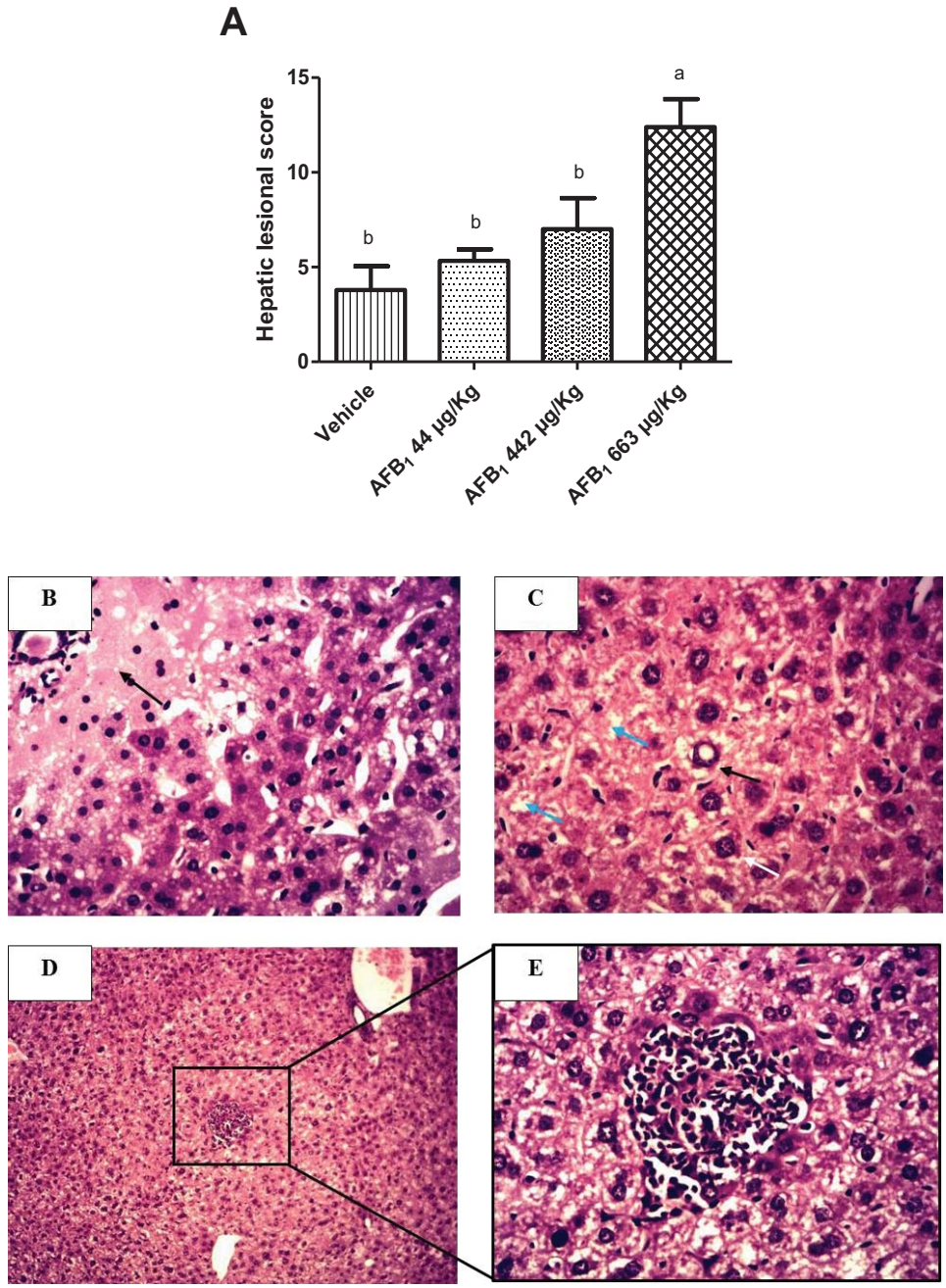

Figure 2. Effect of aflatoxin B_1 in liver mice contaminated with 44 µg, 442 µg and 663 µg of AFB_1/Kg b. w. after five days post-aflatoxin exposure. A) Lesional score. Data was expressed as mean ± SD, $n = 5$. Means without a common letter were statistically significant ($p < 0.05$) by the Tukey's Test. The liver lesions observed in animals treated with 663 µg of AFB_1/Kg were B) focus necrosis hepatocytes (arrow), HE, 40x; C) vacuolar hepatocyte degeneration [nuclear (black arrow) and cytoplasmic (blue arrow)] and megalocytosis (white arrow), HE, 40x; and D) e E) centrolobular inflammatory infiltrate, HE, 10x; e 40x, respectively.

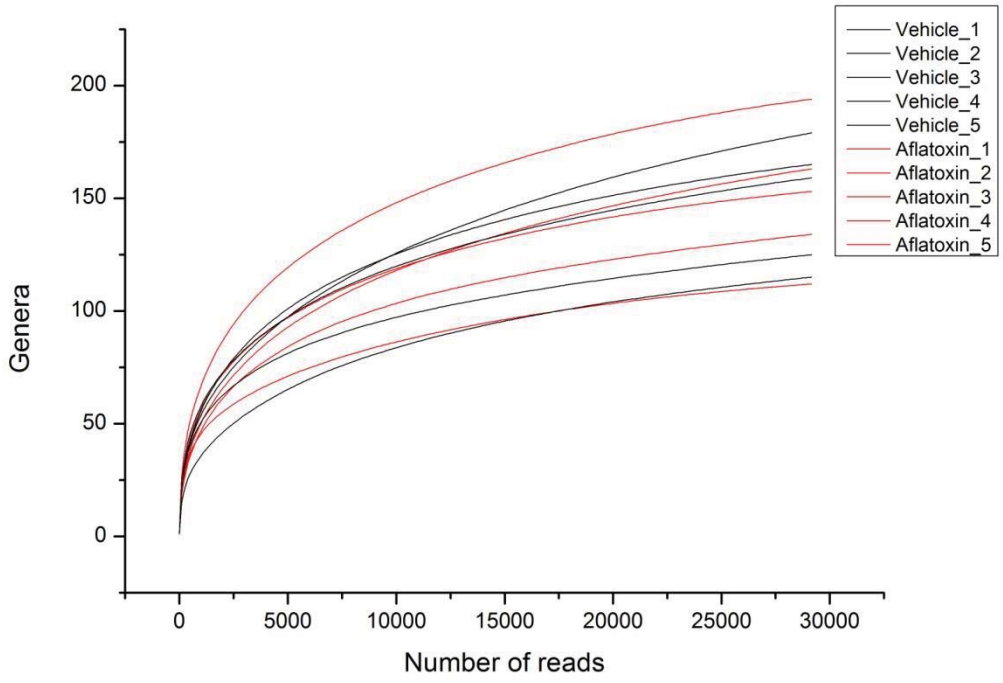

Figure 3 Rarefaction curves representing the richness of gut community of each animal treated with 663 µg of AFB_1/kg of body weight (*n* = 5, red line) compared to the vehicle (*n* = 5, black line) group after five days post-aflatoxin exposure

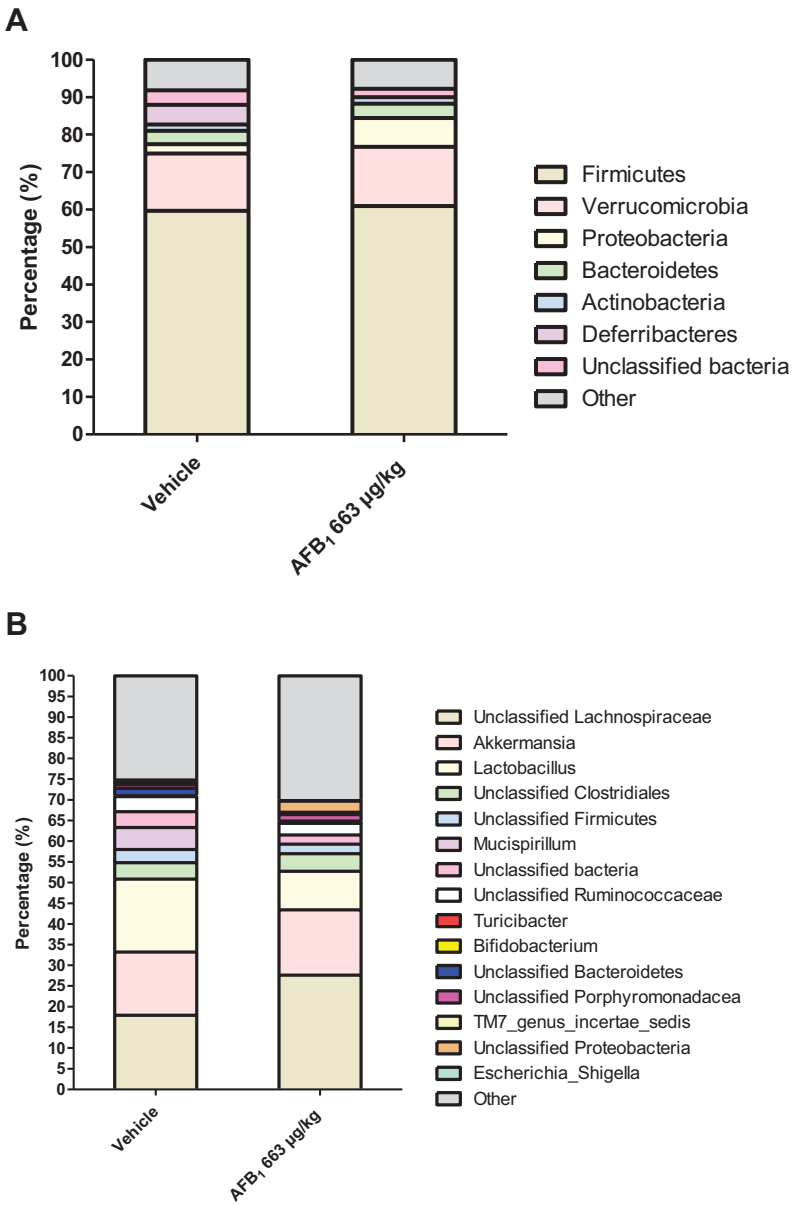

Figure 4. Effect of aflatoxin B_1 on relative abundance of predominant bacteria at the A) phylum and B) genus levels in vehicle and 663 µg of aflatoxin B_1/kg b. w. group after five days post-aflatoxin exposure

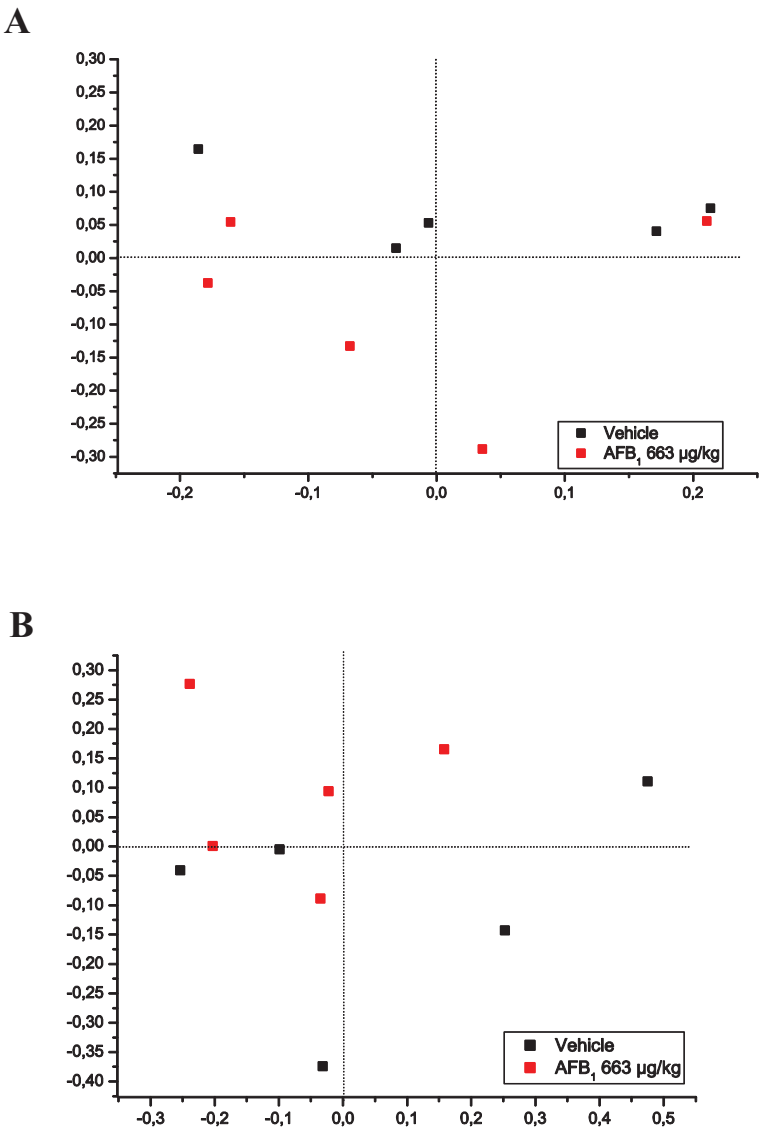

Figure 5. Principal coordinate analysis (PCoA) of bacterial communities present in feces of each mice treated with aflatoxin B_1 (n = 5, each red point represents one animal) and vehicle group (n = 5, each black point represents one animal). PCoA of A) bacterial membership addressed by the Classic Jaccard analysis and B) bacterial communities structure addressed by the Yue-Clayton analysis.

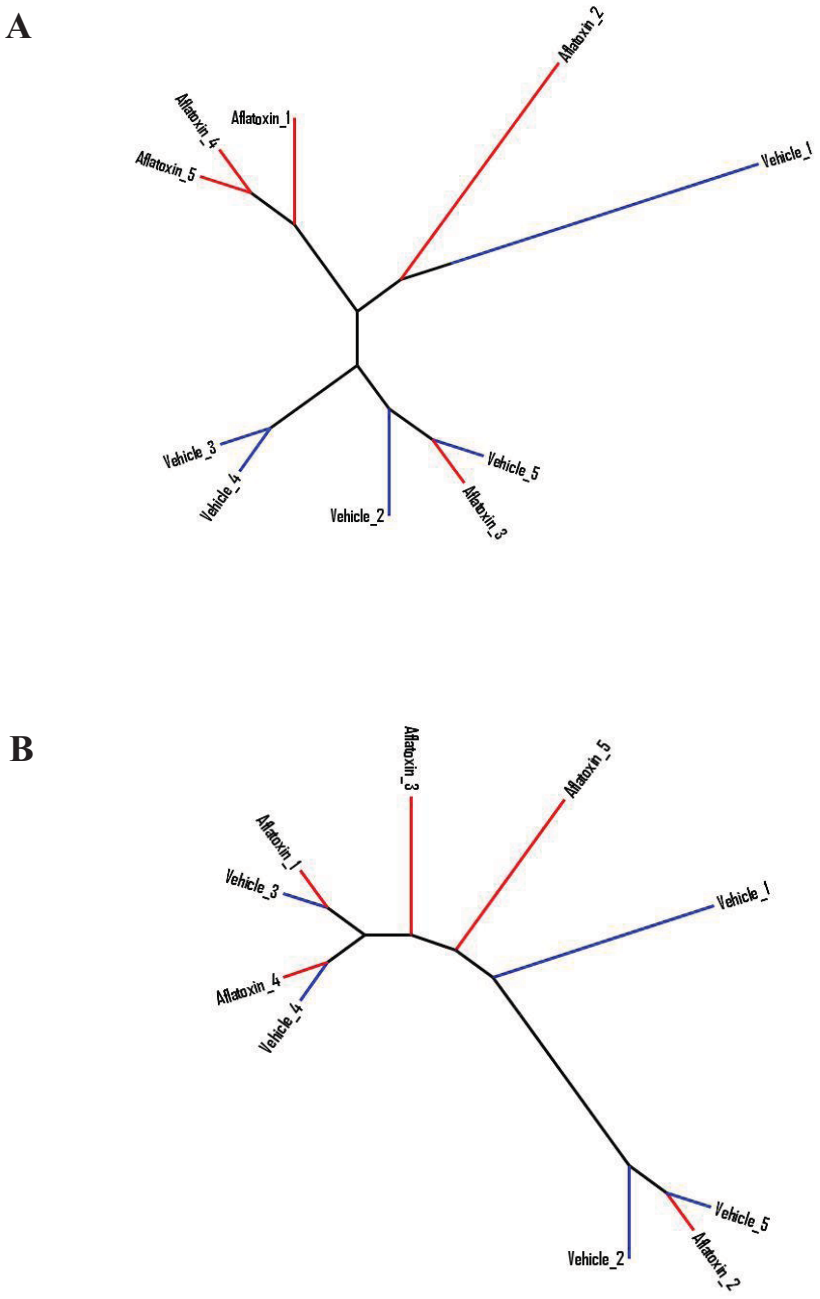

Figure 6. Dendogram of bacterial communities present in feces of each mice treated with aflatoxin B_1 (n = 5, red) and vehicle group (n = 5, blue). Dendogram of A) bacterial membership addressed by the Classic Jaccard analysis and B) bacterial communities structure addressed by the Yue-Clayton analysis.

Manuscrito 4:

Effect of single oral subclinical doses of aflatoxin B_1 on bone marrow cells and lymphoproliferative response to mitogen in C57Bl/6 mice

8. MANUSCRITO 4: EFFECT OF SINGLE ORAL SUBCLINICAL DOSES OF AFLATOXIN B_1 ON BONE MARROW CELLS AND LYMPHOPROLIFERATIVE RESPONSE TO MITOGEN IN C57BL/6 MICE

8.1 ABSTRACT

Aflatoxin B_1 (AFB_1) has a potent carcinogenic and immunossupressive effect, produced as secondary metabolite mainly by *Aspergillus flavus* and *A. parasiticus*. It is found in both food and feed resulting in human and animal risk. The aim of the present study was to investigate the effects of single oral dose of AFB_1 in the liver function, bone marrow cells and splenic lymphoproliferation response to mitogens in mice. A total of 25 male C57Bl/6 mice were allocated in five groups. Three groups were treated with a single oral dose of AFB_1 (44 μg, 442 μg and 663 μg of AFB_1/kg of b. w.) on the first day and they were euthanized on the 5th day. Two control groups were treated only with water and vehicle (saline:ethanol, 95:5). Liver function was assessed by analysis of biochemical serum levels (ALT, γ-GT, total protein), splenic lymphoproliferative response to mitogens (concanavalin A, ConA; lipopolysaccharide, LPS) by MTT test and bone marrow cells count (leukocytes, megakaryocytes, red blood cells). There was a significant suppression of the lymphoproliferative response to ConA-stimulated lymphocytes with 663 μg of AFB_1/kg of b. w. ($p < 0.05$). There was an increase in leukocytes count from dose of 44 μg of AFB_1/kg of b. w, and red blood cells and megakaryocytes at 663 μg of AFB_1/kg of b. w. was observed, but did not produce any significant changes in biochemical parameters. In conclusion, single oral subclinical dose of AFB_1 exposure can induce increase in bone marrow of leukocytes, red blood and megakaryocytes, and interfere on splenic lymphoproliferation in C57Bl/6 mice.

Keywords: hematopoietic system; leukocytes; liver; inflammatory response; immunosuppression; mycotoxin

8.2 INTRODUCTION

Aflatoxins (AFs) are mycotoxins produced by *Aspergillus flavus* and *A. parasiticus* that contaminate agricultural commodities during harvest and post-harvest conditions. Humans and animals health issue caused by ingestion of food contaminated with AFs have been considered a permanent risk and a worldwide problem (Picinin et al., 2013; Santili et al., 2015). Among AFs, aflatoxin B_1 (AFB_1) is normally predominant and considered the most toxic analogue (Liang et al., 2015).

Ingestion of AFB_1-contaminated products can result in immunossupressive, teratogenic, mutagenic and carcinogenic effects. International Agency for Research on Cancer classified AFB_1 as Group 1, *i.e*., carcinogenic to humans (IARC, 2012; Diao et al., 2013). Further, many immunotoxic effects of AFB_1 had been described, ranging from innate immunity or antigen presenting cells (Mehrzar et al., 2014; Mohammadi et al., 2014, Bianco et al., 2012) to adaptive immunity; reducing the number of circulating lymphocytes, inhibition of lymphocytes blastogenesys, cytokine expression, in animals of various species (Bianco et al., 2012, Greiner et al., 2013).

The AFs are highly liposoluble facilitating their absorption from the gastrointestinal and respiratory tract and reach the bloodstream, and subsequently different tissues and organs (IARC, 2002). As bone marrow is vascularized by blood, these AFs can get up to the bone marrow, which contains high levels of multiple immune cell subsets with important and unique functionalities (Zhao et al., 2012). Also, myelopoieses, erythropoiesis and megakaryocytopoiesis all occur primarily within the bone marrow. According to Bianco et al. (2012) and Greiner et al., (2013), AFB_1 alters the ability of cells from the bone marrow to form myeloid and erythroid colonies. There are only few studies about AFB_1 effect on the bone marrow, most of them shows its effect on peripheral blood cells (Donmez et al., 2012;

Lala et al., 2015; Mahfouz, Sherif, 2015). Moreover, in *in vivo* experimental models, animals have been exposed to acute dose or chronic AFB_1 dose but for an extend period to find different effects and impact of AFB_1 exposure on animals (Ilic et al., 2010; Abdulrazzaq et al., 2011; Jha et al., 2013; Mulder et al., 2015). The present study investigated the effects of single oral subclinical dose of AFB_1 (44, 442, 663 µg/kg b. w.) on the hematopoietic system in bone marrow and immune response in C57Bl/6 mice.

8.3 MATERIAL AND METHODS

8.3.1 Aflatoxin B_1 standard

The AFB_1 standard from *Aspergillus flavus* (A663, Sigma, USA) was quantified according to Instituto Adolfo Lutz (IAL, 2008). The molar absorptivity of AFB_1 considered for calculating AFB_1 concentration in methanol was 21,800 at 360 nm.

8.3.2 Aflatoxin B_1 dose and exposure time criteria

The lowest AFB_1 dose (44 µg/kg of body weight, b.w.) was calculated based on maximum levels allowed in both rice and bean which is 5 µg/kg (EC, 2010; ANVISA, 2011) and it was considered average consumption of bean (183 g/day) and rice (160 g/day) as traditional diet in Brazil (IBGE, 2011). As the highest dose of AFB_1 (663 µg/kg of b. w.), it was based on chronic AFB_1 dose used in other studies (Jha et al., 2013; Feitah et al., 2014). The time length of aflatoxin exposure was choosen to determine if even at short period of time it can be detected some effect.

8.3.3 Animals, housing and experimental design

A total of 25 male C57Bl/6 mice (10 weeks-old, average weight: 22.55 ± 0.89 g) were obtained from University of São Paulo – Ribeirão Preto City, Brazil. The C57Bl/6 mice were

chosen for this study as the strain is most susceptible to the acute effect of aflatoxin B_1 (Almeida et al., 1996). The mice were acclimatized for three weeks and housed in polyethylene boxes on a bedding of wood shavings, maintained under standard conditions of temperature about 25 °C with regular 12h light/ 12h dark cycle. All mice were given standard rodent pelleted food and water *ad libitum*.

The experimental design used in this study was randomized with five repetitions (each animal represented one repetition) for each group. Group 1 was untreated control mice; Group 2 received only the vehicle (saline:ethanol, 95:5) on the first day; Group 3, received a single dose of 44 µg of AFB_1/kg b. w. on the first day; Group 4 received a single dose of 442 µg of AFB_1/kg; b. w. and Group 5 received a single dose of 663 µg of AFB_1/kg b. w. The AFB_1 was administered by oral gavage (0.1 mL each 10 g of body weight), wherein AFB_1 was suspended in saline:ethanol (95:5). During 5 days of experiment period, the behavior and feces of animals was observed. After 5 days the animals were weighed, bleed, euthanized, and their organs (liver, spleen, kidney) were removed and weighed. Biochemical parameters were analyzed in serum, lymphoproliferation assay was assessed in spleen and it was held total count of bone marrow cells. This study was approved by the Committee of animal ethics of State University of Londrina (n° 26362.2014.65).

8.3.4 Biochesmitry and bone marrow cells

The ALT and γ-GT enzymes, and total protein level in serum was carried out by Dimension® Clinical Chemistry System (Siemens, USA).

The bone marrow cells were collected from the femur by inserting a syringe needle into one end of the bone and flushing with 0.1 M PBS, pH 7.4. The megakaryocytes, total white and blood cell populations from bone marrow cells were counted manually in a Neubauer chamber. Neutrophils subpopulation was evaluated on Giemsa stained smears.

8.3.5 Lymphoproliferation Assay

Spleen cells from all mice were removed aseptically and the erythrocytes lysed with trisammonium chloride solution. In 96-well flat-bottom culture plates, 100 µL of the spleen cells ($1x10^5$ cells/mL) of each mice were cultured in duplicate wells in RPMI 1640 medium (containing L-glutamine, 10% fetal calf serum), in the presence or not of 0.5 µg/mL of lipopolysaccharide (LPS) (Sigma, USA) or 3.5 µg/mL concanavalin A (ConA) (Gibco, USA). The cells were cultured for 84 h at 37°C with 5% CO_2, and then 100 µL of RPMI medium and 10 µL of 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyltetrazolium bromide (5 mg/mL MTT in PBS, Sigma) were added in each well, and plates were further incubated for 4h at 37°C. After formazan crystals were solubilized by adding 200 µL of dimethyl sulfoxide (DMSO). The optical density was measured by an ELISA microplate reader (iMarkTM, Bio-Rad, USA) at 550 nm, and the proliferation index (P. I.) calculated as the duplicate of stimulated cells/nonstimulated cells (Massuda et al., 2011).

8.3.6 Statistical analysis

Data from biochemical parameters, bone marrow cells count and lymphoproliferative assay were analyzed using Statistica 10.0 (Stat Soft Inc.), and were presented as mean ± standard deviation. Before the analysis, homogeneity of variances (Levene´s Test) and data normal distribution (Shapiro-Wilk's Test) were done. The one-way analysis of variance (ANOVA) followed by the Tukey's Test were performed, and p value < 0.05 was considered as significant.

8.4 RESULTS

8.4.1 Absolute body and organs weight of mice after aflatoxin B_1 exposure

Oral administration of AFB_1 in mice did not decrease the body weight gain when compared with the untreated control group, the initial and final body weight was 22.55 ± 0.89 g and 21.45 ± 1.32 g, respectively ($p > 0.05$). Moreover, it was not observed alterations in kidney, liver and spleen weight in animals treated with AFB_1 in relation to the control group ($p > 0.05$, data not shown).

8.4.2 Evaluation of the effect of aflatoxin B_1 in ALT, γ-GT and total protein levels in mice serum

Oral administration of AFB_1 did not produce any significant change in ALT and γ-GT serum enzymes and protein levels ($p > 0.05$) (Table 1). The untreated control group showed ALT, γ-GT, and protein levels of 26.67 ± 9.37 U/L, 6.83 ± 0.75 U/L and 5.63 ± 0.49 g/dL, respectively. The animals treated with 663 μg of AFB_1/kg showed levels for the same parameters of 22.80 ± 3.70 U/L, 7.00 ± 2.00 U/L and 5.46 ± 0.30 g/dL, respectively. Also the vehicle group treated with saline:ethanol (95:5) presented similar results with control treated only with water ($p > 0.05$).

8.4.3 Effects of aflatoxin B_1 on lymphoproliferation assay

Significant suppression of the proliferative response was observed in Con A-stimulated lymphocytes with 442 and 663 μg of AFB_1/ kg of b. w ($p < 0.05$). The control animals showed lymphoproliferation index of 1.21 ± 0.14, while the experimental animals treated with 442 μg of AFB_1/kg of b. w. dose was 0.94 ± 0.07, and with 663 μg of AFB_1/kg of b. w. was 0.97 ± 0.01. However, no significant depression of the proliferative assay was observed

in LPS-stimulated lymphocytes when the data were compared with the control animals (Figure 1).

8.4.4 Effects of aflatoxin B_1 on total count of bone marrow cells

It was observed an increase in bone marrow leucocytes (neutrophils), red blood, and megakaryocytes cells counts when compared with control group (Figure 2). The animals treated with 44, 442 and 663 μg of AFB_1/kg of b. w. showed 6.79 x 10^6, 10.53 x 10^6 and 10.23 x 10^6 of leucocytes cells/mm^3, which the neutrophils counts were 5.6 x 10^3, 9.30 x 10^3 and 1.02 x 10^4 cells/mm^3 (Figure 2A-B). It was observed an increased count of red blood and megakaryocytes cells in animals treated with AFB_1 when compared to control group ($p < 0.05$), animals treated with 663 μg of AFB_1/kg of b. w. showed counts of 6.27 x 10^3 red blood cells/mm^3 and 2.33 x 10^4 megakaryocytes cells/mm^3 (Figure 2C-D).

8.5 DISCUSSION

Among mycotoxins, AFs are the major concern worldwide in terms of risk to human and animal health. In this study, the animals treated with AFB_1 showed no changes in the body weight as well as kidney, liver and spleen when compared to control animals group (data not shown). Therefore the single dose until 663 μg AFB_1/kg of b. w. exposure did not affect the body and organs weight of C57Bl/6 mice considered as the strain most susceptible to the acute effect of aflatoxin B_1 (Almeida et al., 1996).

As shown in Table 1, the exposure of mice to single dose of AFB_1 did not have a major impact on the biochemical parameters investigated. Such results are consistent with those found by Baptista et al. (2008), who fed Albino rats with 400 μg of AFB_1/kg of b. w. during 28 days and did not find significant differences in the activities of ALT, AST, ALP, γ-GT enzymes and albumin levels. Almeida et al. (1996) reported that intoxicated C57Bl/6

mice presented high levels of alkaline phosphatase in serum at 24 and 72 hours after AFB_1 treatment ($p > 0.05$), but not at 168 hours. Hepatocellular ALT activity in serum increases within 12 hours, with peaks in 1-2 days (Haschek et al., 2010). In this study, the increase levels of hepatic enzymes was not detected possibly because biochemical parameters were analyzed after five days after AFB_1 exposure.

In chronic and subclinical aflatoxicosis cases, changes in biochemical and hematological parameters, as well as deleterious effects on the hematopoietic system in bone marrow has been observed before clinical symptoms develop (Donmez et al., 2012; Bbsosa et al., 2013; Lala et al., 2015). In this study, longer period post exposure time was not included, because the subclinical status may be kept during 28 days, considering the Baptista et al. (2008) results.

Concerning the hematopoietic system in the bone marrow cells, it was detected increased leukocytes number from the 44 µg AFB_1/kg of b. w. and red blood cells and megakaryocytes only with 663 µg AFB_1/kg of b. w. (Figure 1), suggesting that the neutrophils are the primary bone marrow cells affected by AFB_1. Aflatoxicosis cause lymphocytopenia and monocytopenia, whereas the leukocytes percentage increase suggesting that AFB_1 elicited an inflammatory response (Donmez et al., 2012). According to Zhao et al (2012), bone marrow is a large pool for the mature neutrophils, the majority of neutrophils are reserved in the bone marrow and a large amount of neutrophils can be mobilized rapidly in response to infection and stress. This increased production in bone marrow could be related to some inflammatory response in target organs.

In a study conducted by Reddy and Sharma (1989), the BALB/c mice received 700 µg of AFB_1/kg of b. w. orally every other day for four weeks, a similar AFB_1 dose was used in present study (663 µg/kg of b. w.). The longer treatment time with AFB_1 triggered a decrease in peripheral leukocytes number. In this study, the amount of circulating

neutrophils was not investigated, but it is possible that bone marrow is conducted to increase of neutrophils production for inflammatory process.

Several researchers have reported effects of AFB_1 treatment in the reduction on total red blood cell (Abdel-Wahhab et al., 2002; Mahfouz, Sherif, 2015). This decrease in the hematological parameters may be due to many factors such as inhibition of protein synthesis as enzymes involved in heme biosynthesis, decrease of the total iron binding capacity, and the hematopoietic cellular defects (Donmez et al., 2012; Mahfouz, Sherif, 2015). According to Verma & Raval (1991), higher AFB_1 concentrations in the blood may cause self-destruction of red blood cells, i.e., hemolysis, whereas lower AFB_1 concentration will result in morphological alterations and a natural elimination of red blood cells by its destruction at reticuloendothelial tissues. In this present study, this fact could explain the increase in red blood cells produced in the bone marrow, since the body is trying to recover the homeostasis.

Likewise, the AFB_1 exposure at 663 µg/kg of b. w. dose increased the megakaryocytes count. Thousands of platelets are formed from a single megakaryocytes cell, which the most known function is coagulation following vascular injury. Abnormalities in the platelet production process can result in clinically significant disorders, such as thrombocytopenia can lead to inadequate clot formation and increased risk of bleeding, and thrombocythemia can heighten the risk for thrombotic events (Patel, 2005). In addition, platelets are involved in diverse processes such as triggering inflammation and participating in the immune response. Besides the signaling of toll-like receptors, platelets can release a variety of cytokines and chemokines, providing fast mechanisms in the acute inflammatory response (Oliveira et al., 2013).

Bone marrow has been long thought to be a hematopoietic organ, but Zhao et al, (2012) suggested that bone marrow also may play a key role in immunity. Then, future studies on AFB_1 exposure in animals with identification of various T cell populations in the

bone marrow would be important to elucidate whether this organ can also be involved in systemic immunomodulation in aflatoxicosis.

In addition, it has been reported that AFB_1 has a selective effect on cell-mediated immunity with a relatively low effect on the humoral immune system (Li et al., 2014). Reddy & Sharma (1989) reported inhibitory effects of AFB_1 on LPS- and ConA-stimulated lymphoblastogenesis in animals exposed to low repeated doses of AFB_1 and were primarily on T-cell functions. These findings are in accordance with the present study, which lymphoproliferative response in mice spleen cells was suppressed by AFB_1 in the presence of ConA, a lectin widely used as a polyclonal T cell activator, but not with LPS.

In this study, by using single dose of AFB_1 in C57Bl/6 mice, it was possible to detect changes in leucocytes and neutrophils, red blood and megakaryocytes cells count in bone marrow, and splenic lymphocytes proliferation in 5 days. The short period and a subclinical dose used in this experiment represent a fast and simple system of aflatoxin evaluation. Posteriorly, it can be applied for other bioassay system, as for example, the evaluation of antioxidants to inhibit the toxic effects of aflatoxin.

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Table 1. Effects of aflatoxin B_1 on ALT, γ- GT and total protein levels in mice serum after five days of oral single dose of aflatoxin B_1

Group	Parameters		
	ALT (U/L)	**γ - GT (U/L)**	**Total protein (g/dL)**
Control	26.67 ± 9.37[a]	6.83 ± 0.75 [a]	5.63 ± 0.49 [a]
Vehicle	43.25 ± 15.52 [a]	6.5 ± 0.71 [a]	5.46 ± 0.54 [a]
AFB_1 44 µg/Kg	37.33 ± 8.64 [a]	6.3 ± 1.03 [a]	5.83 ± 0.27 [a]
AFB_1 442 µg/ Kg	28.33 ± 7.23 [a]	5.50 ± 0.71 [a]	5.12 ± 0.34 [a]
AFB_1 663 µg/Kg	22.80 ± 3.70 [a]	7.00 ± 2.00 [a]	5.46 ± 0.30 [a]

Means without a common letter were statistically significant ($p < 0.05$) by the Tukey's Test. Reference values for mice serum enzymes are 23.00 ± 4.92 units of ALT/L, 7.57 ± 4.2 units of γ-GT/L, and total protein of 5.07 ± 0.2 g/dL (Almeida et al., 1996; Fernández et al., 2010)

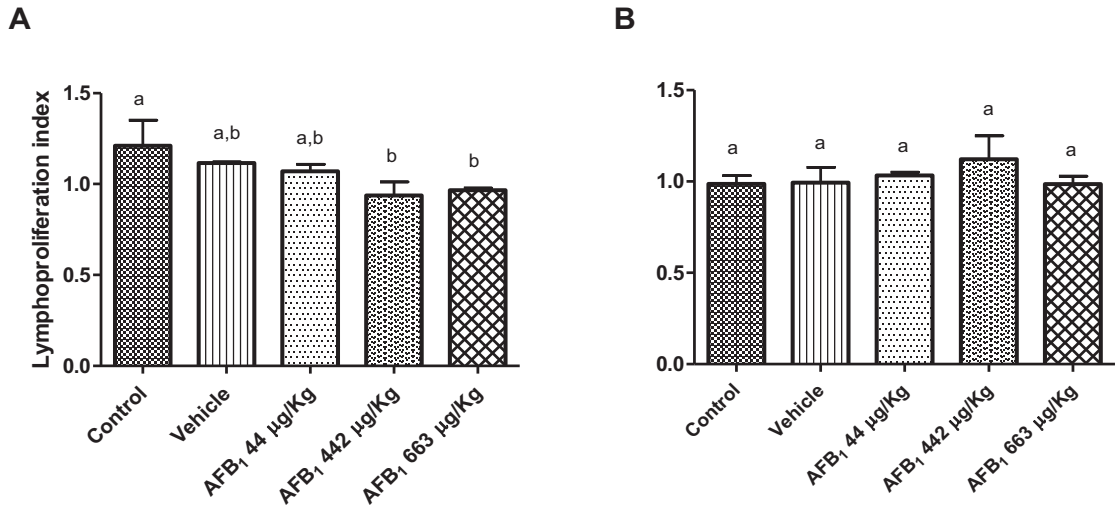


Figure 1. Effect of aflatoxin B_1 on proliferative response in mice spleen cells in the presence of A) concanavalin A and B) lipopolysaccharide after five days post-aflatoxin treatment. The significance levels are from data of experimental animal when compared to control group. Data was expressed as mean ± SD of the proliferative index (OD of the test well/OD of control well), $n = 5$. Means without a common letter were statistically significant ($p < 0.05$) by the Tukey's Test.

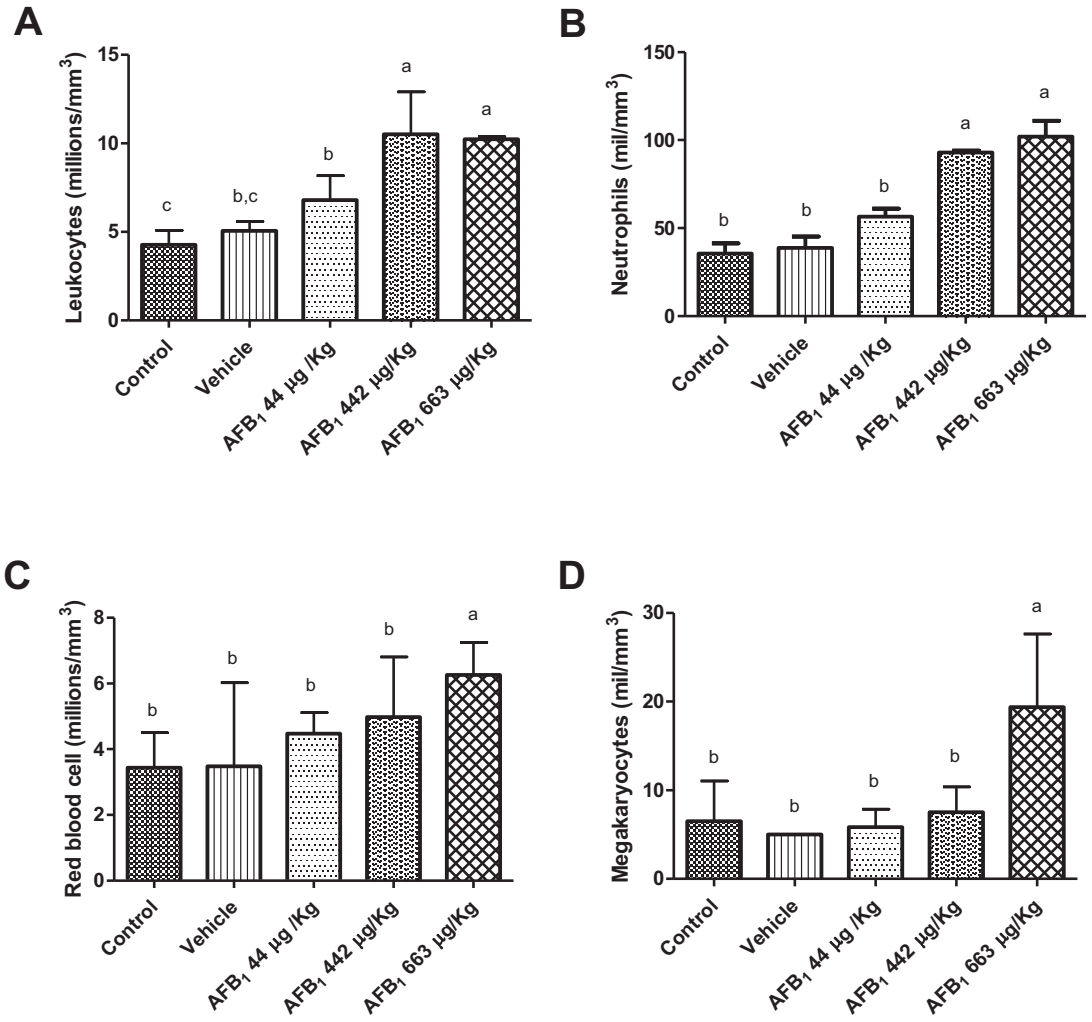


Figure 2. Effect of aflatoxin B_1 on the number of A) leukocytes, B) neutrophils C) red blood cells, and D) megakaryocytes in bone marrow cells after five days post-aflatoxin exposure. Data was expressed as mean ± SD, $n = 5$. Means without a common letter were statistically significant ($p < 0.05$) by the Tukey's Test.

9. CONCLUSÕES GERAIS

i. O consumo de alimentos que são possíveis fonte de contaminação por aflatoxina B_1 e M_1 pelas mães lactantes no Norte do Paraná, Brasil, é baixo quando comparado com a média de consumo da América Latina.

ii. O método de extração de aflatoxina M_1 em leite materno e leite em pó infantil apresentou recuperação variando de 110,25 a 88,35%, e 98,21 a 71,67%, respectivamente.

iii. A contaminação por aflatoxina M_1 foi detectada em níveis baixos (n.d. - 0,025 ng de AFM_1/g) em leite materno no Norte do Paraná.

iv. A contaminação por aflatoxina M_1 foi detectada em níveis baixos no leite em pó infantil comercializado no Norte do Paraná, sendo que 18,8% (n = 3) das amostras excederam contaminação máxima permitida pela legislação da Comunidade Européia (0,025 ng de AFM_1/g).

v. Pelo cálculo de Ingestão Estimada Diária para infantes (0 a 12 meses) do gênero masculino e feminino, conclui-se que a exposição de infantes à aflatoxina M_1 é baixa.

viii. O peso corpóreo e de órgãos (fígado, baço e rim) de camundongos expostos a doses de 44 ou 442 ou 663 µg de AFB_1/kg não é alterado.

ix. A exposição oral de camundongo C57Bl/6 a AFB_1 induz lesão hepática nas doses subclínicas, com maior alteração na dose de 663 µg/kg.

x. Camundongos C57Bl/6 expostos a ingestão oral de AFB_1 na dose de 663 µgFB_1/kg induz modulação de citocina hepática.

xi. Camundongos C57Bl/6 expostos a ingestão oral de AFB_1 nas doses de 442 e 663 µgFB_1/kg induziram supressão na resposta linfoproliferativa ao mitógeno ConA.

xii. Camundongos C57Bl/6 expostos a ingestão oral de AFB_1 na dose de 663 µgFB_1/kg apresentaram alteração no sistema hematopoiético da medula óssea.

xiii. Camundongos C57Bl/6 expostos a ingestão oral de AFB_1 na dose de 663 µgFB_1/kg apresentaram alteração na microbiota bacteriana intestinal com aumento na família Lachnospiraceae residente no intestino grosso.

10. CONSIDERAÇÃO FINAL

Há uma necessidade constante de proteger a saúde de seres humanos e animais limitando sua exposição às micotoxinas. Muitos países implementaram legislações ou sugerem níveis máximos tolerados de micotoxinas em alimentos por causa do significante impacto na saúde e perda na economia. Apesar de muitos anos de pesquisa e a introdução de boas práticas na cadeia alimentar de várias commodities, as micotoxinas continuam sendo um problema permanente e mundial, principalmente em países em desenvolvimento. É importante lembrar que se faz necessário a implementação de uma legislação mais rigorosa no Brasil, e uma rigorosa e contínua rotina de monitoramento.

APÊNDICES

APÊNDICE 1

Immunoaffinity column (IAC) confection for clean-up of milk samples

. *Hybridoma cell culture and monoclonal antibody purification*

Hybridoma AM.3 secreting monoclonal antibody for AFM_1 derived from SP2/0-Ag14 myeloma cell line and BALB/c spleen produced by Okumura *et al.* (1993) was used. The IC_{50} value of AM.3 to AFM_1 was 6.1 pg/mL, and its cross-reactivities (%) were 0.2 (AFM_2), 0.002 (AFG_1), <0.0006 (AFG_2), 0.003 (AFB_1), <0.0006 (AFB_2), <0.006 (AFcol I), <0.0006 (AFcol II), 0.026 (AFP_1) and <0.0006 (AFQ_1).

Hybridoma stored in liquid nitrogen (-185°C) was thawed and the cells were centrifuged with 10 mL of Hybridoma- Serum Free Medium (H-SFM Complete DPM, cat n° 12300-067, lote 1218743, Gibco, USA) at 1400 rpm for 6 minutes. The supernatant was discarded and added 4 mL of GIT Medium (398-00515, Wako) in 24 wells plate (1 mL/well), the medium was changed to H-SFM and the cells were transferred to 75 cm^2 bottle (Figure 1). The cultivation was proceeded at 37°C, 5% CO_2 (CO_2 Cell Culture Incubator, MCO-18 AIC(UV), Sanyo). When the cells concentration was 8 to 10 x 10^5 cells/mL, 120 mL of H-SFM was added in 75 cm^2 bottle, gently mixed and then, 12 mL of H-SFM was placed in 10 dishes. After 7-10 days, the supernatant in dishes was filtered through five filter types (Figure 2). The supernatant was filtered and purified by protein G column (HiTrap^{TM} Protein G HP, Lot 710348, 1 x 5 mL, GE Healthcare, Sweden) (Figure 3). After purification, antibody purified was dialyzed against Phosphate Buffer Saline (PBS- 3x) and kept at -30°C.

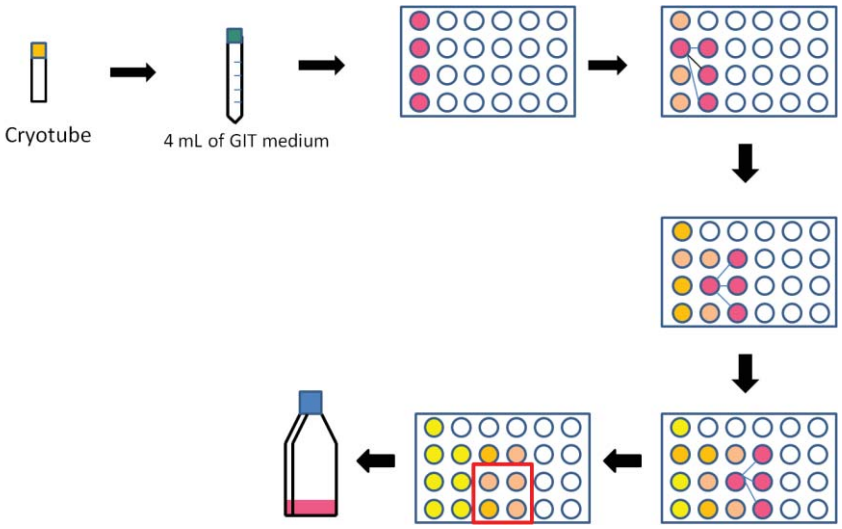


Figure 1. Cryotube contained 0.5 mL of cells was thawed in water bath at 37°C. Cells were suspended in 4 mL of GIT medium and placed in 24 wells plate (1mL/well). One well was choosen to expand in more three wells and when cells concentration were 1 to 10x10^5 cells/ mL, they were transferred to 75 cm^2 bottle.

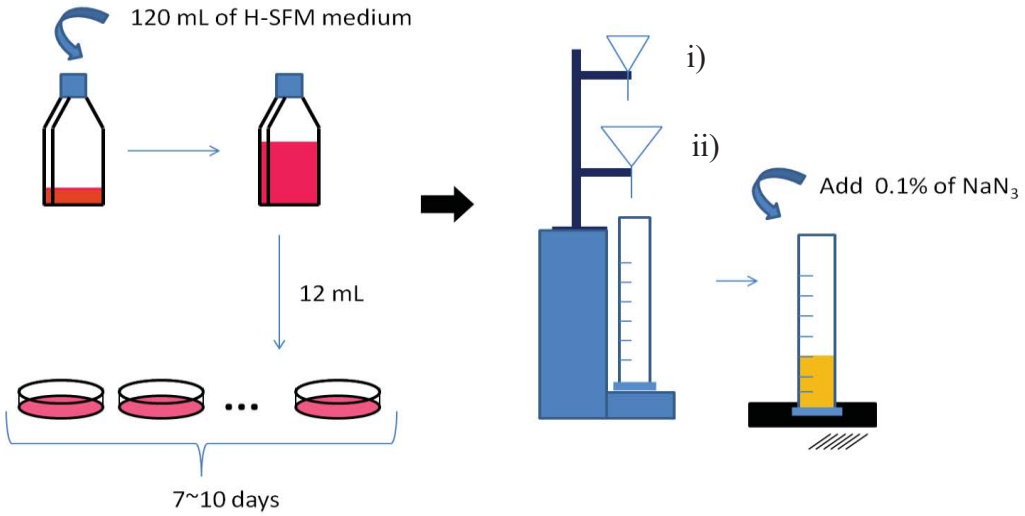


Figure 2. Three 75 cm^2 bottles were kept during almost two months of Hybridoma culture. Every two days when the cells concentration was 8 to 10 x10^5 cells/mL, 120 mL of H-SFM was added in each bottle, mixed and placed in dishes (12 mL per dish). After 7-10 days, the supernatant was filtered by five types of filter: i) Filter Paper Qualitative (131, 185 mm, Advantec, Japan); ii) Filter Paper Quantitative Ashless (5C, 185 mm, Advantec, Japan); iii) Glass Fiber Filter (GF-75, 45 mm, Advantec, Japan); iv) Cellulose Acetate (C300A047A, 47 mm, 3µm, Advantec, Japan); v) Cellulose Acetate (C020A047A, 47 mm, 0.2 µm, Advantec, Japan). In this picture shows only the use of the two first filters.

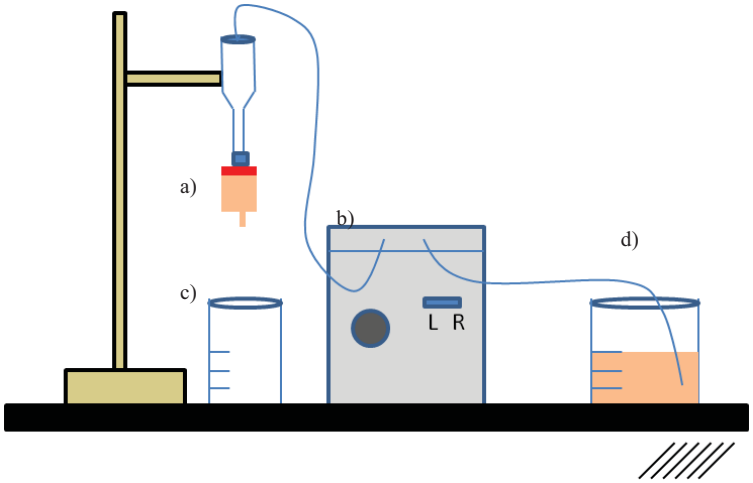


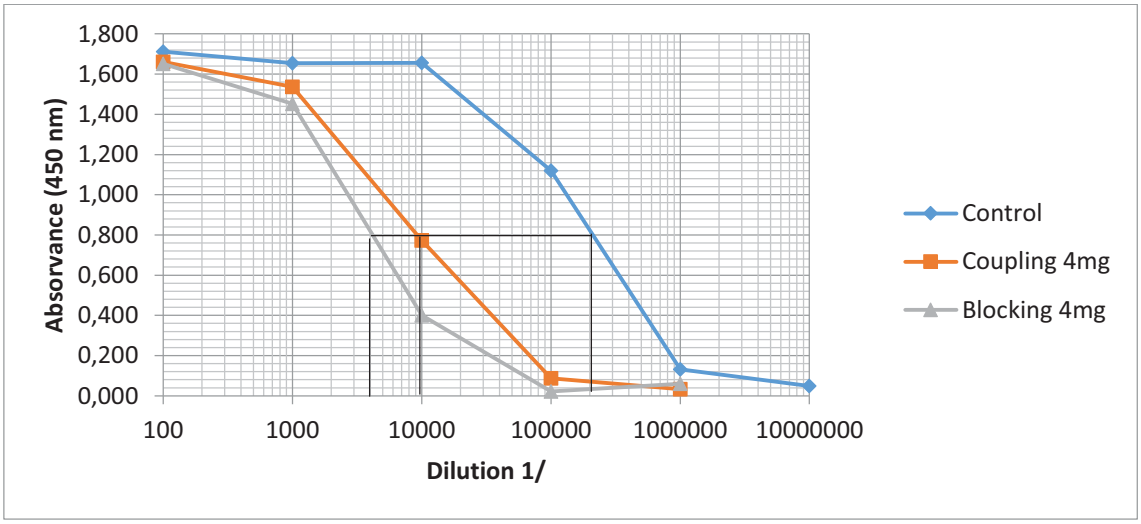
Figure 3. The supernatant was purified by Protein G Column using i) 35 mL of 20 mM $NaPO_4$ Buffer pH 7.0 (2 drops/second); ii) 300 mL of supernatant (1 drop/second); iii) 50 mL of 20 mM $NaPO_4$ Buffer pH 7.0 (2 drops/second); iv) 15 mL of Glycine-HCl pH 2.7 (1 drop/second) Each 1 mL was collected in tubes containing 30 µL of 1 mol/L Tris-HCl pH 9.0; v) 20-40 mL of 20 mM $NaPO_4$ Buffer pH 7.0 (2 drops/second); vi) 20 mL of 20% ethanol in $NaPO_4$ (2 drops/ second); vii) The eluate was read by spectrophotometer at 280 nm (UV-1800, Shimazdu, Japan); viii) dialysis against Phosphate Buffer Saline (PBS) for 3 times. a) Protein G Column; b) Mini pump (Perista mini-pump, SJ-1211, ATTA); c) Discard; d) Supernatant filtered.

. Indirect-ELISA (i-ELISA) for development of IAC

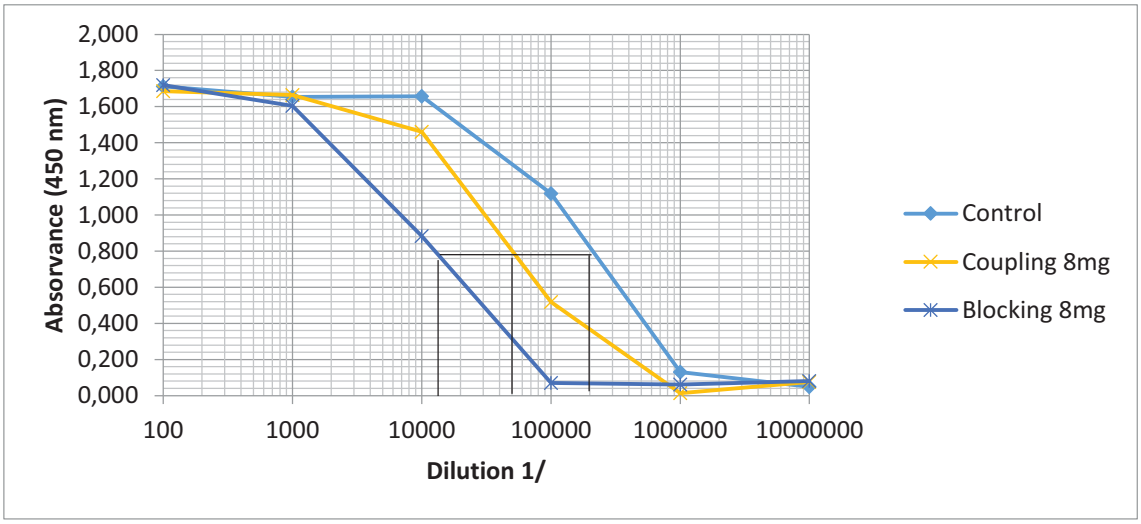
The 96-wells microplate (Nunc^{TM}, Denmark) was coated with 100 µL of AFM_1-BSA conjugate (200 ng/mL- A-6412, Sigma) in 0.01 mol/L PBS pH 7.4 and incubated for 16 h at 4 °C. After washing (3 times) with 0.05 % PBS-Tween (PBST), the wells were blocked with 125 µL of 0.1% ovalbumin solution and incubated for 1 h at 25 °C. After washing (3x) with 0.05% PBST, 100 µL of solutions steps of IAC development (Control, Post-coupling and Post-blocking step) diluted (1/100; 1/1,000; 1/10,000; 1/100,000; 10,000 000) in 0.05% PBST was added. The incubation was realized for 45 min at 25 °C; then, the microplate was washed (3x) with 0.05% PBST, 100 µL of horseradish peroxidase (HRP)-labeled goat anti-mouse H^+L chain specific (SouthernBiotech, USA) was added and incubated for 45 min at 25 °C. Posteriorly, the microplate was washed (6x) with 0.05 % PBST, and 100 µL of tetramethylbenzidine solution was added, followed by incubation for 15 min at 25 °C. The reaction was stopped by adding 50 µL of 1 mol/L H_2SO_4, and the absorbance was measured at 450 nm (NJ-2300, Immunomini, Japan).

. Immunoaffinity Column confection

The coupling efficiency of 4 mg or 8 mg mAb anti-AFM_1 coupled to Affi-gel for IAC confection was evaluated by i-ELISA. For 4 mg of mAb anti-AFM_1 coupled to 1 mL of Affi-gel, 93% was coupled to the gel (3.7 mg of mAb anti-AFM_1), an amount of 5% was not coupled in Post-coupling step and 2% in Post-blocking step (Figure 4-a). For 8 mg of mAb anti-AFM_1, 25% and 7.5% were not coupled in Post-coupling and Post-blocking step, respectively, it means loss of 2.6 mg of mAb anti-AFM_1 (Figure 4-b). Due to higher loss of mAb anti-AFM_1, IAC was prepared with 4 mg of mAb anti-AFM_1/mL of Affi-gel was choosen to proceed the recovery test.



(a)



(b)

Figure 4. Ab activity from post-coupling and post-blocking by i-ELISA for 4 mg of mAb anti-AFM_1 mL^{-1} of gel (a), 93% of mAb (3.7 mg) was coupled to the gel. An amount of 5% was not coupled in post-coupling step (10,000/200,000= 0.05=5%) and 2% was not coupled in Post-blocking step (4,000/200,000=0.02=2%). For 8 mg of mAb anti-AFM_1 mL^{-1} of gel (b), 25% was not coupled in Post-coupling step (50,000/200,000= 0.25= 25%) and 7.5% was not coupled in Post-blocking step (15,000/200,000=0.075=7.5%). Therefore, 67.5% of mAb (5.4 mg) was coupled to the gel.

APÊNDICE 2

Stability test of AFM_1 in different reagents

1st step: Solvent choice for AFM_1 elution in the IAC

The solvent chosen to keep stability of AFM_1 was based on following tests. Initially, AFM_1 stability was tested in 5 mL of seven different reagents (methanol, methanol:acetonitrile, acetonitrile, ethanol, acetone, isopropanol, acetonitrile:water), using polypropylene (PP) or glass tube to dry AFM_1 standard in centrifugal evaporator (Table 1). The higher AFM_1 stability was in acetone (n 7 and 8) and CH_3CN (n 11 and 12) for both types of tubes.

Table 1. AFM_1* stability in different reagents and proportions

n	Reagent	Tube	Stability (%)
1	Methanol	Glass	25.7 ± 6.9
2	Methanol	PP	14.8 ± 2.6
3	Methanol:acetonitrile (1:4;v/v)	Glass	30.0 ± 8.0
4	Methanol:acetonitrile (1:4;v/v)	PP	28.4 ± 0.8
5	Ethanol	Glass	13.2 ± 2.0
6	Ethanol	PP	7.9 ± 0.3
7	Acetone	Glass	99.1 ± 5.1
8	Acetone	PP	101.9 ± 0.5
9	Isopropanol	Glass	19.8 ± 15.4
10	Isopropanol	PP	14.7 ± 2.7
11	Acetonitrile	Glass	101.9 ± 2.3
12	Acetonitrile	PP	95.1 ± 0.6
13	Acetonitrile:water (1:1, v/v)	Glass	25.8 ± 7.0
14	Acetonitrile:water (1:1, v/v)	PP	14.7 ± 2.7

* 2.5 ng of AMF_1 (50 µL of 50 ng/mL)

2nd step: AFM_1 recovery test in the IAC

Acetone and acetonitrile were choosen to proceed elution step in the IAC. Recovery test was done applying 10 mL of PBS 0.01 mol/L pH 7.4 in the IAC, followed by 2.5 ng of AFM_1 in 5mL of

PBS 0.01 mo/L pH 7.4 (0.5 ng/mL), washed with 5 mL of PBS 0.01 mol/L pH 7.4 and 5 mL of water. AFM_1 was eluted with acetone or acetonitrile, and then dried. The conditions tested were amount of mAb anti-AFM_1 for IAC confection, AFM_1 dissolved in different reagents to proceed recovery test, different reagents for elution step and two types of tubes for drying samples.

. Amount of Ab in the IAC

i) 3.7 mg of mAb anti-AFM_1 mL^{-1} of gel / 0.3 mL of gel per IAC;

ii) 3.7 mg of mAb anti-AFM_1 mL^{-1} of gel / 0.5 mL of gel per IAC;

iii) 5.4 mg of mAb anti-AFM_1 mL^{-1} of gel / 0.3 mL of gel per IAC;

. AFM_1 in:

i) PBS;

ii) Acetonitrile:PBS (1:4);

iii) Acetonitrile:PBS (1:9);

iv) Acetonitrile:PBS (3:7)

. Solvent to elution step:

i) acetone;

ii) acetonitrile,

. Volume of solvent to elution step

i) 3 mL

ii) 5 mL

iii) 8 mL

. Glass or PP tube to dry the sample

AFM_1 recovery was low in all cases (11 to 69%). Probably, the problem was in the elution step, because a large volume of solvent is required to elute (at least 8 mL of solvent).

3rd step: Choosing another solvent for AFM_1 elution in the IAC

AFM_1 stability was tested in 2 mL of different proportions of methanol and acetonitrile (1:1; 1:4; 1:9), using PP tube and dried in centrifuged evaporator (Table 2).

Table 2. AFM_1* stability in different proportions of methanol:acetonitrile

n	Reagent	Stability (%)
15	Methanol:acetonitrile (1:1; v/v)	10.8 ± 5.62
16	Methanol:acetonitrile (1:4; v/v)	55.2 ± 2.51
17	Methanol:acetonitrile (1:9; v/v)	95.5 ± 3.03

* 2.5 ng of AMF_1 (50 µL of 50 ng/mL)

Amount of 2 mL of methanol:acetonitrile (1:9; v/v) shows high stability of AFM_1, but it is not with 5 or 10 mL of methanol:acetonitrile (1:9; v/v) (Table 3). The drying time could be the problem.

Table 3. AFM_1* stability in different volume of methanol:acetonitrile

n	Reagent	Volume (mL)	Stability (%)
18	Methanol:acetonitrile (1:9;v/v)	2	95.8 ± 1.84
19	Methanol:acetonitrile (1:9;v/v)	5	82.7 ± 8.68
20	Methanol:acetonitrile (1:9;v/v)	10	46.6 ± 27.97

* 2.5 ng of AMF_1 (50 µL of 50 ng/mL)

4th step: The use of rotary evaporator to dry samples

AFM_1 stability was tested in rotary evaporator to decrease time to dry. In all cases, the AFM_1 stability was higher than 91.4%, even in large volumes of solvent (Table 4). Therefore, rotary evaporator was used to dry samples for analysis by HPLC.

Table 4. AFM_1* stability in methanol and/or acetonitrile

n	Reagent	Volume (mL)	Stability (%)
21	Methanol	2	91.4
22	Methanol:acetonitrile (1:4;v/v)	2	94.1
23	Methanol:acetonitrile (1:9;v/v)	2	100
24	Methanol:acetonitrile (1:9;v/v)	5	93.8
25	Methanol:acetonitrile (1:9;v/v)	10	94.1

* 2.5 ng of AMF_1 (50 µL of 50 ng/mL)

APÊNDICE 3

Method evaluation for breast milk and infant powdered milk analysis by HPLC

The Limit of Detection was considered as the lowest AFM_1 concentration that could be reliably detected (LOD, signal:noise ratio, > 3:1) and Limit of Quantification (LOQ) was signal:noise ratio, >10:1. The recovery rate was evaluated using reconstituted BM and IPM blank as sample in three fortified levels (0.025, 0.05 and 0.5 ng of AFM_1/g) in triplicate. The repeatability measured by relative standard deviation was checked from replicates of reconstituted BM and IPM samples in the recovery study. Linearity was determined from 3 calibration curves with 5 points between 0.025 and 5 ng of AFM_1/mL As results, the Limit of Detection (LOD, signal:noise ratio, > 3:1) and Limit of Quantification (LOQ, signal:noise ratio, >10:1) were 0.003 ng/g and 0.016 ng/g for BM, and 0.004 ng/g and 0.021 ng/g for IPM, respectively. The equation was $y = 1090832.98\ x - 10692.71$, with coefficient of determination (R^2) greater than 0.99. The mean recovery of spiked BM ($n = 3$) at levels of 0.025, 0.05 and 0.5 ng of AFM_1/g were 110.25%, 97.21% and 88.35%, respectively. The recovery test values for IPM at the same levels were 98.21%; 77.92% and 71.67% (Table 5).

Table 5. Recovery test for breast milk and infant powdered milk

AFM_1 (ng/g)	BREAST MILK			INFANT POWDERED MILK		
	% Recovery	Mean ± SD (ng/g)	% RSD	% Recovery	Mean ± SD (ng/g)	% RSD
0.025	106.26 116.61 107.87	110.25 ± 5.57	5.05	98.03 91.88 104.73	98.21 ± 6.43	6.54
0.05	96.14 96.03 99.48	97.21 ± 1.96	2.02	79.2 76.53 78.03	77.92 ± 1.34	1.72
0.5	87.54 88.04 89.48	88.35 ± 1.01	0.01	72.46 72.19 70.35	71.67 ± 1.15	1.60

BM, LOD: 0.004 ng/mL; LOQ: 0.021 ng/mL
IPM: LOD: 0.003 ng/mL; LOQ: 0.016 ng/mL
SD: Standard Deviation; RSD: Relative Standard Deviation

ANEXOS

ANEXO 1

Universidade Estadual de Londrina

PARANÁ

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 5231

Parecer CEP/UEL:	159/2012
CAAE:	06371612.5.0000.5231
Data da Relatoria:	29/04/2013
Pesquisador(a):	Eiko Nakagawa Itano
Unidade/Órgão:	CCB – Programa de Pós-Graduação em Patologia Experimental

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"**Determinação de niveis de OTA e AFM1 em amostras de leite materno, urina e soro humano**".

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá ser encaminhado ao CEP/UEL relatório final da pesquisa, conforme prevê a Resolução 196/96 do Conselho Nacional de Saúde/MS e Resoluções Complementares.

Londrina, 29 de abril de 2013.

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

ANEXO 2

UNIVERSIDADE ESTADUAL DE LONDRINA
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS GRADUAÇÃO EM PATOLOGIA EXPERIMENTAL

QUESTIONÁRIO DE FREQUÊNCIA DE CONSUMO ALIMENTAR

Este questionário é composto por duas partes:
1) Na primeira parte você deve responder questões sócio-culturais.
2) Na segunda parte responda sobre seu consumo alimentar. Entre parênteses você vai encontrar a medida caseira comumente utilizada.

1) Questões sócio-culturais

Nome:_____
Idade:_____Sexo: ( ) masculino ( ) feminino
Profissão: _____Peso (Kg):_____
Altura (m): _____ Cidade onde nasceu:_____
Cidade onde mora:_____
Bairro:_____
Região da cidade onde mora: ( ) Norte ( ) Sul ( ) Leste ( ) Oeste

Local de compra dos alimentos consumidos:
( )feira ( ) mercado ( ) outros_____
Em qual região da cidade o alimento consumido é comprado? ( ) Norte ( ) Sul ( ) Leste ( ) Oeste

Quantas vezes na semana você costuma fazer refeições fora de casa?_____
Realiza quais refeições fora de casa? ( ) almoço ( ) janta
Quais em casa? ( ) café da manhã ( ) almoço ( ) lanche
( ) café da tarde ( ) janta

Está realizando dieta no momento? ( ) não ( ) sim
Realiza alguma atividade física? ( ) não ( ) sim. Qual? _____
Você fuma? ( ) não ( ) sim
Consome bebidas alcoólicas? ( ) não ( ) sim
Apresenta algum problema de saúde relacionado à alimentação? ( ) não ( ) sim. Qual?
( ) colesterol elevado ( ) intolerância a lactose
( ) triglicerídeos elevados ( ) diabetes
( ) hipertensão ( ) outras_____
Já apresentou algum problema renal? ( ) não
( ) sim. Qual?_____

Já apresentou algum problema no fígado? ( ) não
( ) sim. Qual?_____

Toma algum medicamento? ( ) não ( ) sim. Qual? _____

2) Consumo alimentar

✓ Marque com um **X** a frequência com que você consome os seguintes alimentos. Marque somente um **X** em cada linha.

Alimentos	Não consumo	Consumo						
		2 vezes ao dia	1 vez ao dia	3 a 4 vezes na semana	1 a 2 vezes na semana	a cada 15 dias	1 vez ao mês	1 vez ao ano
Café torrado em grão ou moído (1 colher de sopa~10g/ 50 mL)								
Café solúvel (1 colher de café ~5g/ 50mL)								
Cerveja (copo de 200 mL)								
Uva (unidade)								
Uvas passa (1 colher sopa)								
Suco de uva (copo de 200 mL)								
Vinho tinto (copo de 200 mL)								
Vinho rosado (copo de 200 mL)								
Vinho branco (copo de 200 mL)								
Leite em pó (1 colher de sopa ~15g/copo de 200mL)								
Leite fluido (copo de 200 mL)								
Iogurtes (copo de 200 mL)								
Queijos (fatia ~20g)								
Pimenta, páprica e **noz-moscada** (1 colher café)								
Castanha-do-Brasil (1 colher de sopa)								
Nozes, pistaches, avelãs e **amêndoas** (1 colher de sopa)								
Amendoim (1 colher sopa)								
Pé-de-moleque e paçoca (1 unidade)								
Milho em grão inteiro, amassado ou moído (1 colher de sopa)								

✓ Quais alimentos ingeridos um dia anterior à coleta do leite?

Alimento	**Quantidade** (colher, fatia, xícara, copo)
1.	
2.	
3.	
4.	
5.	
6.	
7.	
8.	
9.	
10.	
11.	
12.	
13.	
14.	
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17.	
18.	
19.	
20.	
21.	
22.	
23.	
24.	
25.	

ANEXO 3

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA N º 207/2014 **Londrina, 18 de Dezembro de 2014.**

Prezada Pesquisadora,

A CEUA/UEL, reunida em 02 de Dezembro de 2014, avaliou o pedido de complementação intitulado **"Avaliação do efeito protetivo de resveratrol contra exposição aguda de AFB_1 em camundongos C57BL/6"**, processo CEUA **nº26362.2014.65**, referente ao projeto de pesquisa intitulado **"Análise do efeito imunopatológico de micotoxinas (OTA, AFB_1, AFM_1 e FB_1) em camundongos recém-natos"**, processo CEUA **nº23165.2012.57** aprovado sob ofício circular de **nº252/12,** pesquisa do Centro de Ciências Biológicas sob sua responsabilidade, a solicitação foi **aprovada** entendendo-se que os princípios éticos postulados pelo Conselho Nacional de Controle de Experimentação Animal estão respeitados.

Serão utilizados 96 camundongos C57BL/6 adultos com 25 g provenientes da Universidade de São Paulo, Ribeirão Preto – SP. O procedimento aprovado tem o objetivo de avaliar o efeito do resveratrol sobre as alterações imunopatológicas (injuria hepatocelular, análise histopatológica e imunohistoquímica) na exposição aguda de aflatoxina B1 (AFB_1) em camundongos. O projeto está previsto para ser desenvolvido em 11 meses.

Cumpre orientar que caso pretendam-se quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação da CEUA/UEL anteriormente à execução das modificações. Sem mais para o momento, subscrevo-me. Cordialmente,

Waldiceu Ap. Verri Junior

Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador da CEUA/UEL

Ilma. Sra.
Profa. Dra. Eiko Nakagawa Itano
Coordenadora do Projeto
Departamento de Ciências Patológicas
Centro de Ciências Biológicas
Com cópia para Sra Égle Maria de Sousa (Chefe da DCA/PROPPG), Chefe do Departamento de Ciências Patológicas e Diretor(a) do Centro de Ciências Biológicas.

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), km 380 - Fone (043) 3371-4000 PABX - Fax 3328-4440 - Caixa Postal 10.011 - CEP 86057-970 – Internet http://www.uel.br
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