UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

OCORRÊNCIA NATURAL DE MICOTOXINAS EM MILHO (*Zea mays* L.) E SUA INFLUÊNCIA NO DESEMPENHO DE FRANGOS DE CORTE

TESE DE DOUTORADO

Maurício Schneider Oliveira

Santa Maria, RS, Brasil

2014

OCORRÊNCIA NATURAL DE MICOTOXINAS EM MILHO (*Zea mays* L.) E SUA INFLUÊNCIA NO DESEMPENHO DE FRANGOS DE CORTE

Maurício Schneider Oliveira

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Doutor em Ciência Animal

Orientador: Prof. Carlos Augusto Mallmann

Santa Maria, RS, Brasil

2014

FICHA CATALOGRÁFICA

Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Schneider Oliveira, Maurício
Ocorrência natural de micotoxinas em milho (Zea mays L.) e sua influência no desempenho de frangos de corte / Maurício Schneider Oliveira.-2014.
63 p.; 30cm

Orientador: Carlos Augusto Mallmann
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós-Graduação em Medicina Veterinária, RS, 2014

1. Fungos 2. Micotoxinas 3. Segurança alimentar 4. Desempenho 5. Frangos de corte I. Mallmann, Carlos Augusto II. Título.

Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós-Graduação em Medicina Veterinária

A Comissão Examinadora, abaixo assinada,
aprova a Tese de Doutorado

OCORRÊNCIA NATURAL DE MICOTOXINAS EM MILHO (*Zea mays* L.) E SUA INFLUÊNCIA NO DESEMPENHO DE FRANGOS DE CORTE

elaborada por
Maurício Schneider Oliveira

Como requisito parcial para obtenção do grau de
Doutor em Ciência Animal

COMISSÃO EXAMINADORA

Carlos Augusto Mallmann, Dr. (UFSM)
(Presidente/Orientador)

Jaqueline Garda Buffon, Dr. (FURG)

Plínio Barbarino Junior, Dr. (BIOMIN)

Luciane Minetto, Dr. (UCS)

Carlos Alberto Araújo de Almeida, Dr. (UFSM)

Santa Maria, 8 de agosto de 2014.

AGRADECIMENTOS

Aos professores Carlos Augusto Mallmann e Paulo Dilkin a amizade, o aprendizado e o voto de confiança em meu trabalho.

À Dra. Jaqueline Garda Buffon sempre solícita em diversas etapas deste trabalho.

Aos membros da banca avaliadora Dr. Plinio Barbarino, Dr. Luciane Minetto, Dr. Ana Flávia e Dr. Diego de Souza as suas valiosas contribuições.

Ao amigo Dr. Ricardo H. Rauber a amizade, auxílio estatístico e qualificadas sugestões ao trabalho.

A todos os amigos do Laboratório de Análises Micotoxicológicas que me auxiliaram, apoiaram e participaram da realização de experimentos, discussões e momentos de descontração. Em especial, agradeço aos amigos Adriano O. Mallmann, Alexandre Rocha, Andressa C. L. Diel, Carlos A. A. de Almeida, Fabiana P. Fontoura e Marília M. May, o envolvimento e colaboração direta na realização deste trabalho. Aos amigos Alexandro Marchioro, Fernanda Dambrós e Mayara Mattei a amizade e apoio ao longo desta trajetória.

Aos amigos do Instituto de Soluções Analíticas e Microbiológicas, especialmente André L. Mallmann, Luis A. de Cezaro, Diego Mallmann, Diego Sturza, Leandro Z. Giacomini a amizade, e envolvimento na realização da experimentação animal.

Aos amigos do *Analytikzentrum* da Universidade de Viena, especialmente Dr. Rudolf Krska, Dr. Michael Sulyok, Dr. Rainer Schuhmacher, Dr. Franz Berthiller, Elisabeth Varga, Benedikt Warth, Viktoria Preiser, Antonia Tacconi e Renate Hörmann, a amizade, o auxílio e apoio na realização do estágio de doutorado sanduíche.

Ao Programa de Pós-Graduação em Medicina Veterinária a oportunidade de fazer parte de seu corpo discente.

A Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) o apoio financeiro ao longo de todo período de estudo.

A minha família, o apoio e incentivo durante toda minha vida.

Muito Obrigado!

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

OCORRÊNCIA NATURAL DE MICOTOXINAS EM MILHO (*Zea mays* L.) E SUA INFLUÊNCIA NO DESEMPENHO DE FRANGOS DE CORTE
AUTOR: MAURÍCIO SCHNEIDER OLIVEIRA
ORIENTADOR: CARLOS AUGUSTO MALLMANN
Santa Maria, 8 de agosto de 2014.

Fungos podem ser encontrados em toda a natureza e são de conhecida ocorrência na cultura do milho. Aqueles que possuem a capacidade de produzir substâncias tóxicas para humanos e animais, a partir de seu metabolismo secundário, denominam-se fungos toxígenos e os metabólitos produzidos são denominados micotoxinas. Devido a presença de diversas espécies fúngicas toxígenas na lavoura de milho, a ocorrência de um grande número de metabólitos é esperada. Além de ocorrerem em sua forma nativa, micotoxinas podem ocorrer na forma designada mascarada, resultado de alterações em sua estrutura ou de reações químicas com constituintes de plantas, tornando-as indetectáveis para as metodologias de análise convencionalmente empregadas. Em consequência aos prejuízos que as micotoxinas podem causar a saúde humana e animal, legislações estabelecendo limites máximos permitidos de contaminação em alimentos tem sido propostas, porém restritas a um pequeno número de micotoxinas, em parte devido a limitações analíticas e parte em decorrência da escassez de dados de ocorrência natural destes contaminantes na forma nativa e mascarada. Para avaliar a ocorrência de micotoxinas no milho, 148 amostras de milho foram coletadas nos estados pertencentes a região sul do Brasil. Todas as amostras estavam contaminadas com pelo menos 10 metabólitos fúngicos e a maior co-ocorrência detectada em uma mesma amostra foi 51 de compostos. Em todas as amostras de milho analisadas, foram detectadas pelo menos duas micotoxinas listadas na legislação brasileira (fumonisina B_1 e fumonisina B_2). Além da pesquisa das formas nativas de micotoxinas, foram realizadas análises para determinação de fumonisinas na forma mascarada. Setenta e duas amostras de milho foram analisadas e a concentração de fumonisinas mascaradas encontrada foi até duas vezes maior do que a concentração de fumonisinas na forma nativa. Ademais, uma correlação positiva (R=0.97) foi encontrada entre a concentração de fumonisinas na forma nativa e mascaradas. Baseado nesta correlação, foi gerado um modelo matemático de predição para estimativa da concentração de fumonisinas totais, baseado na medida de fumonisinas na forma nativa. Como consequência a constatação da co-ocorrência a diversas micotoxinas, o desempenho de frangos de corte submetidos a dietas contaminadas com materiais de cultivo fúngico foi avaliado. Peso corporal e conversão alimentar foram significativamente alterados aos 21 dias de experimento no grupo que recebeu dieta contendo maiores concentrações de ácido fusárico. As metodologias analíticas aplicadas permitiram uma primeira abordagem para estudo da co-ocorrência de micotoxinas na sua forma nativa e na forma mascarada em amostras de milho naturalmente contaminadas, coletadas na região sul do Brasil. O efeito negativo sobre o desempenho de frangos de corte observado, demonstrando que a co-ocorrência de micotoxinas do gênero *Fusarium* tem real impacto na avicultura e requer maior atenção das partes envolvidas.

Palavras-chave: Fungos. Micotoxina. Segurança alimentar. Desempenho. Frangos de corte.

ABSTRACT

Doctoral Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

NATURAL MYCOTOXIN OCCURRENCE IN MAIZE (*Zea mays* L.) AND INFLUENCE ON THE PERFORMANCE OF BROILER CHICKENS

AUTHOR: MAURÍCIO SCHNEIDER OLIVEIRA
ADVISER: CARLOS AUGUSTO MALLMANN
Santa Maria, August 8th, 2014.

Fungi can be found everywhere in nature and are known occur in maize. Those which have the ability to produce substances toxic to humans and animals, from its secondary metabolism, are named toxigenic fungi and the metabolites produced are called mycotoxins. Due to the presence of various toxigenic fungal species in maize crop, the occurrence of a large number of metabolites is expected. Besides occur in native forms, mycotoxins can occur in the form called masked, a result of changes in structure or chemical reactions with constituents of plants, making them undetectable to the analytical methodologies conventionally employed. Due to the damage that mycotoxins can cause to human and animal health, laws establishing maximum permitted levels of contamination in food has been proposed, but restricted to a small number of mycotoxins, partly due to analytical limitations and partly in consequence of the lack of database of natural occurrence of these contaminants in the native and masked forms. To evaluate the occurrence of fungal metabolites in maize, 148 samples were collected in the South region of Brazil. All samples were contaminated with at least 10 fungal metabolites and the higher co-occurrence detected in the same sample was 51 metabolites. In all maize samples analyzed were detected at least two mycotoxins adressed by Brazilian law (fumonisin B_1 and fumonisin B_2). Besides the research of the native forms of mycotoxins, analysis for masked fumonisin were performed. Seventy two maize samples were analyzed and masked fumonisin concentration was found up to two times greater than the concentration of fumonisins in native form. Furthermore, a positive correlation ($R = 0.97$) was found between the concentration of native and masked fumonisin. Based on this correlation, a mathematical prediction model to estimate the concentration of total fumonisins, based on the concentration of native fumonisins was generated. After knowing the co-occurence of several fungal metabolites in maize, the performance of broilers chickens submitted to diets with fungal culture material was evaluated. Body weight and feed conversion were significantly altered after 21 days of experiment in the group receiving diet containing higher concentrations of fusaric acid. The analytical methodologies enabled a first approach to study the co-occurrence of mycotoxins in their native and masked forms in maize naturally contaminated, collected in the South region of Brazil. The negative effect on the performance of broilers chickens, demonstrating that the co-occurrence of *Fusarium* mycotoxins have real impact in poultry and requires greater attention of the parties involved.

Key words: Fungi. Mycotoxin. Food Safety. Performance. Broiler chickens

LISTA DE FIGURAS

ARTIGO 1.

Figura 1 – Relation between contamination of free fumonisins and total fumonisins in maize samples analyzed by LAMIC/UFSM. * Average of contamination of free fumonisins (FB1 + FB2) ** Total fumonisins concentration estimated by predictive model. 46

Figure 2 – Correlation between free and total fumonisins concentration based on determination of hydrolyzed forms in raw maize samples. The found equation for this correlation was: Total fumonisins = (0.8583+0.5615*Free fumonisins)ˆ2; R = 0.97. 46

LISTA DE TABELAS

ARTIGO 1
Tabela 1 – Frequency of regulated mycotoxin contamination in maize samples by state 30

Tabela 2 – Natural co-contaminating, regulated mycotoxins in maize samples 30

Tabela 3 – Natural co-contaminating fungal and bacterial non-regulated metabolites in maize from the South region of Brazil 31

ARTIGO 2
Tabela 1 – Retention time and compound dependent parameters for LC-ESI-MS/MS analysis of free and hydrolyzed fumonisins 47

ARTIGO 3
Tabela 1 – Análises micotoxicológicas dos materiais de cultivo fúngico 58

Tabela 2 – Parâmetros de desempenho avaliados em frangos de corte submetidos a dietas contendo diferentes materiais de cultivo fúngico 58

Tabela 3 – Análises sorológicas realizadas em frangos de corte submetidos a dietas contendo material de cultivo fúngico 58

LISTA DE ABREVIATURA E SIGLAS

3-NPA	3-Nitropropionic Acid
Ac.DON	Acetyl-deoxynivalenol
AFB	Aflatoxin B
AFG	Aflatoxin G
AGEITEC	Agência Embrapa de Informação Tecnológica
ANVISA	Agência Nacional de Vigilância Sanitária
ANOVA	Analysis of Variance
AUR	Aurofusarin
AVER	Averufin
AVFV	Averufanin
BEA	Beauvericin
CA	Conversão alimentar
CAD	Collision gas
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEUA	Comissão de Ética no Uso de Animais
CID	Compound Identifier
CLAE	Cromatografia líquida de alta eficiência
CRM	Certified Reference Material
CUR	Curtain gas
CV	Coeficiente de variação
DON	Deoxynivalenol
DNA	Deoxyribonucleic Acid
Dr.	Doutor
EC	European Comission
EFSA	European Food Safety Authority
ESI	Eletrospray Ionization
FAPAS	Food Analysis Performance Assessment Scheme
FB	Fumonisin B
FUA	Fusaric Acid
FUC	Fusarin C
GS1	Nebulizer gas
GS2	Heater gas
HFB	Hydrolized fumonisin B
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
KOH	Hidróxido de Potássio
LAMIC	Laboratório de Análises Micotoxicológicas
LC	Liquid Chromatography
LOD	Limit of detection
LOQ	Limit of quantification
M	Molar
m^2	Metros quadrados
MTL	Maximum Tolerated Limit
mm	Millimeters
MON	Moniliformim
MRM	Multiple Reaction Monitoring
MS/MS	Mass Spectrometry
NID	Nidurufin

NIV	Nivalenol
NOR	Norsolorinic Acid
NRC	National Research Council
PPT	Proteínas plasmáticas totais
REC	Recuperação
SA/SO	Relação média dos níveis de esfinganina/esfingosina no soro
STE	Sterigmatocystin
TCA	Tricarballylic Acid
VER	Versicolorin
µg/kg	Microgram per kilogram
v/v	Volume to volume
ZON	Zearalenone

SUMÁRIO

INTRODUÇÃO 13
ARTIGO 1 - 16
Abstract 17
Introduction 18
Material and methods 19
Results and discussion 21
Conclusion 25
References 26
ARTIGO 2 - 33
Abstract 34
Introduction 35
Material and methods 37
Results and discussion 39
Conclusion 42
References 43
ARTIGO 3 - 48
Abstract 49
Introdução 50
Material e métodos 51
Resultados e discussão 53
Conclusão 55
Bibliografia 56
Carta de aprovação CEUA 59
CONCLUSÕES 60
BIBLIOGRAFIA 62

INTRODUÇÃO

Por um longo período, os fungos foram considerados como vegetais, e somente após o conhecimento de características que os diferenciam das plantas, foram classificados em um reino à parte, denominado *Fungi*. As principais características que diferenciam os fungos de plantas são: ausência de síntese de clorofila, não possuem celulose na constituição da parede celular e são capazes de depositar glicogênio como substância de reserva, ao invés do amido, como ocorre nos vegetais. (GOMPERTZ et al., 2008).

Fungos são ubíquos na natureza e tem evoluído ao longo do tempo para colonizar uma grande variedade de culturas, onde encontram substrato e condições ambientais favoráveis ao seu crescimento (MALLMANN & DILKIN, 2007). O conjunto de reações metabólicas que são essenciais ao crescimento dos fungos, é designado metabolismo primário, enquanto que toda e qualquer atividade metabólica que produza compostos químicos não essenciais ao crescimento fúngico é denominado metabolismo secundário (MAGAN & ALDRED, 2007).

O metabolismo secundário dos fungos é conhecido por produzir uma enorme gama de compostos químicos, alguns úteis ao uso farmacêutico, tendo como exemplos a penicilina e o grupo das estatinas, e outros capazes de produzir efeitos tóxicos a humanos e animais, denominados micotoxinas (MAGAN & ALDRED, 2007). Nem todos os fungos são capazes de produzir micotoxinas e aqueles que possuem esta capacidade são chamados fungos toxígenos (FRISVAD et al., 2007).

Atualmente, centenas de compostos químicos são classificadas como micotoxinas, produzidos principalmente por fungos toxígenos pertencentes ao gênero *Aspergillus*, *Fusarium*, *Penicillium* e *Alternaria* (BENNETT & KLICH, 2003). Cada espécie de fungo toxígeno possui capacidade genética para produzir um perfil de metabólitos secundários em resposta as condições ambientais à que o fungo estiver submetido, dessa maneira, complexas combinações de micotoxinas podem ocorrer na natureza (GRENIER & OSWALD, 2011).

Micotoxinas também podem ocorrer conjugadas a macromoléculas de amido ou proteína, e/ou incorporadas a estruturas das plantas colonizadas por fungos toxígenos (BERTHILLER et al., 2013). Atualmente, o processo de conjugação de micotoxinas na natureza ainda não é totalmente esclarecido, mas acredita-se que enzimas envolvidas no processo de detoxificação das plantas são responsáveis por produzir naturalmente estas formas de micotoxinas (BERTHILLER et al., 2009). Além disso, micotoxinas conjugadas podem ser produzidas de forma não intencional, quando alimentos contaminados forem

submetidos a processos industriais, como aquecimento e fermentação (BERTHILLER et al., 2009).

Quando micotoxinas são ingeridas, por ocasião da alimentação humana ou animal, podem produzir diversos efeitos deletérios a saúde do consumidor e ao desempenho animal (MALLMANN & DILKIN, 2007). Os efeitos tóxicos produzidos pelas micotoxinas, devem-se às diferentes estruturas químicas, aos níveis de contaminação de cada micotoxina existente no alimento, a possível interação toxicológica produzida por diferentes micotoxinas, tempo de consumo do alimento contaminado, e no caso de micotoxinas conjugadas, o risco de que as formas originais de micotoxinas sejam liberadas após digestão no trato gastrointestinal (BERTHILLER et al., 2013; HUSSEIN & BRASEL, 2001). Além disso, os efeitos tóxicos das micotoxinas podem variar entre os organismos vivos, dependendo da espécie afetada, sexo, idade, condições nutricionais e fatores ambientais (MALLMANN & DILKIN, 2007).

A Agência Internacional para Pesquisa sobre Câncer (do inglês, *IARC, International Agency for Research on Cancer*) tem classificado diversas micotoxinas de acordo com seu potencial carcinogênico para humanos. As aflatoxinas, micotoxinas produzidas por fungos pertencentes ao gênero *Aspergillus*, são classificadas no grupo I - compostos carcinógenos para humanos (IARC 2002), já as micotoxinas produzidas por *Fusarium verticillioides*, fumonisina B_1, fumonisin B_2 e fusarina C são classificadas no grupo 2B - compostos possivelmente carcinogênicos para humanos (IARC, 1993). Em animais, micotoxinas podem causar redução no desempenho, causar imunossupressão, afetar órgãos vitais, interferir na capacidade reprodutiva e em casos de intoxicações agudas causar a morte dos animais (BATH et al., 2010). Comparado aos seus efeitos tóxicos individuais, micotoxinas que co-contaminem um alimento podem ter seus efeitos tóxicos aumentados, especialmente em relação ao desempenho animal (SMITH & SEDDON, 1998).

Em consequência aos riscos tóxicos associados as micotoxinas e a possibilidade de alimentos estarem contaminados por diversas metabólitos em concentrações que variam sazonalmente, legislações que estabelecem limites máximos para a presença de micotoxinas em alimentos tem sido propostas, porém restritas a um pequeno número de compostos (BRASIL, 2011).

Os métodos analíticos tradicionalmente utilizados para análise qualitativa e quantitativa de micotoxinas em alimentos, são baseados em imunoensaios e técnicas cromatográficas. Os imunoensaios são na maioria das vezes utilizados para triagem de classes de micotoxinas, devido a sua sensibilidade, rapidez e baixo custo, enquanto as técnicas cromatográficas tem sido a metodologia de escolha para análise de diversas micotoxinas com

variadas estruturas químicas, pertencentes a diversas classes químicas. Diversos métodos analíticos baseados em cromatografia líquida e detecção por espectrometria de massas tem sido desenvolvidos e constantemente atualizados para detectar diferentes classes de micotoxinas concomitantemente. (ABIA et al., 2013; BATH et al., 2010; SULYOK et al., 2006).

Algumas micotoxinas produzidas por fungos toxígenos, possuem limites máximos permitidos estabelecidos por lei e têm sua relevância para humanos e animais descrita e caracterizada ao longo dos últimos anos. No entanto, a segurança alimentar dos alimentos não pode ser garantida apenas pela pesquisa de micotoxinas previstas em legislações. A ocorrência de quadros clínicos e subclínicos têm sido associadas as micotoxinas, mesmo quando concentrações abaixo dos limites permitidos são detectadas. Sendo de presença constante no milho, a ocorrência de micotoxinas com estruturas variadas e em baixas concentrações, interage no organismo vivo exercendo seus efeitos tóxicos.

Diante desta realidade, o objetivo deste trabalho foi o de avaliar a ocorrência natural de micotoxinas em amostras de milho coletadas na região Sul do Brasil e avaliar o efeitos provocados pela intoxicação com micotoxinas, no desempenho de frangos de corte, até os 21 dias de vida.

ARTIGO 1

Natural mycotoxin contamination of maize (*Zea mays* L.) in the South region of Brazil

Maurício S. Oliveira[a], Alexandre Rocha[a], Michael Sulyok[b], Rudolf Krska[b], Carlos A. Mallmann[a]

(Artigo submetido ao periódico *Food Control* – 2014)

[a] Universidade Federal de Santa Maria (UFSM), Laboratório de Análises Micotoxicológicas (LAMIC), CEP 97105 900, Santa Maria, Rio Grande do Sul, Brasil

[b] University Of Natural Resources and Life Sciences, Vienna (BOKU), Department for Agrobiotechnology (IFA-Tulln), Konrad Lorenzstrasse 20, A-3430 Tulln, Austria

* Corresponding author. Tel.: +55 55 3220 8445

E-mail address: mallmann@lamic.ufsm.br (Carlos A. Mallmann)

Natural mycotoxin contamination of maize (*Zea mays* L.) in the South region of Brazil

Maurício S. Oliveira[a], Alexandre Rocha[a], Michael Sulyok[b], Rudolf Krska[b], Carlos A. Mallmann[a] *

[a] Universidade Federal de Santa Maria (UFSM), Laboratório de Análises Micotoxicológicas (LAMIC), CEP 97105 900, Santa Maria, Rio Grande do Sul, Brasil
[b] University Of Natural Resources and Life Sciences, Vienna (BOKU), Department for Agrobiotechnology (IFA-Tulln), Konrad Lorenzstrasse 20, A-3430 Tulln, Austria
* Corresponding author. Tel.: +55 55 3220 8445
E-mail address: mallmann@lamic.ufsm.br (Carlos A. Mallmann)

Abstract

The natural co-occurrence of fungal metabolites in maize samples from the South region of Brazil was studied using an LC-MS/MS based multi-mycotoxin method. All maize samples (n=148) were contaminated with fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2). Aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) were detected in 38 and 11 samples, respectively, while zearalenone (ZON) and deoxynivalenol (DON), which were first regulated in 2014, were found in 110 and 71 samples, respectively. Apart from regulated mycotoxins, a broad range of non-regulated metabolites, from *Aspergillus*, *Fusarium*, *Alternaria*, *Penicillium* and other microbes, were also detected in maize sample. Fusarin C, a possible carcinogenic compound to humans, produced by *Fusarium* species and not addressed by Brazilian legislation, was detected in 54.2% of maize samples. All analysed maize samples were found to be contaminated by at least ten different metabolites, with the largest number of metabolites found in the same sample being 51.

Keywords: mycotoxin, maize, fumonisin, aflatoxin, food safety

Highlights

- The co-occurrence of mycotoxins in maize samples from Brazil was noted;
- Fumonisins B_1 and B_2 were detected in all samples;
- *Aspergillus*, *Fusarium*, *Alternaria*, *Penicillium* and bacterial metabolites were detected.

Abbreviations

AFB_1 aflatoxin B_1; AFB_2 aflatoxin B_2; AFG_1 aflatoxin G_1; AFG_2 aflatoxin G_2; FB_1 fumonisin B_1; FB_2 fumonisin B_2; ZON zearalenone; DON deoxynivalenol.

1. Introduction

The importance of maize (*Zea mays* L.) in southern Brazil is linked to social and economic factors: it is the main source of employment for many families in rural areas (Farias, Robbs, Bittencourt, Andersen, & Corrêa, 2000). Due to its versatility of use, it is used in human consumption in its raw form or in maize-based products, and as the main ingredient in animal feed formulations, mainly for the poultry, swine, and dairy cattle industries (AGEITEC, 2014). Comprised of Rio Grande do Sul, Santa Catarina and Paraná states, the South region of Brazil was responsible for 32.6% of Brazilian maize production during 2012/2013 (CONAB, 2014).

Maize crops are prone to fungal and bacterial disease in the field, after harvest, and also during storage. Due to specific environmental and storage conditions of maize grains, fungi and bacteria may produce mycotoxins and bacterial metabolites, which can induce toxic responses in human and animals after ingestion of a contaminated diet (Queiroz, et al., 2012). Maize grains can be affected by more than one fungus, and each fungus is able to produce several metabolites in the field and during storage; a complex mixture of metabolites may contaminate such maize (Grenier & Oswald, 2011).

Aflatoxins, fumonisins and zearalenone are among the most studied mycotoxins in Brazilian maize due to the effects of each individual toxin: Aflatoxins (AF) are primarily hepatotoxic and are able to induce immunosuppressive, carcinogenic and mutagenic effects. Fumonisins (FB) are known to disrupt sphingolipid metabolism, inducing nephro- and hepatotoxic effects while zearalenone (ZON) is responsible for inducing oestrogenic effects, mainly in swine, after intake of contaminated feed (Richard, 2007).

Furthermore, previous research described the biochemical and physiological damage induced by single or mixtures of mycotoxins. Cortinovis, Caloni, Scheiber, and Spicer (2014), reported that interaction between fumonisin B_1 (FB_1) and ZON can produce additive effects. Bracarense et al., (2012) found several types of interactions (synergistic, additive, less than additive and antagonistic), depending on the parameters and segments assessed in piglets fed

a diet co-contaminated with fumonisins and deoxynivalenol (DON). In a field study of dairy cows submitted to diets co-contaminated with ZON and DON, depressed milk production, and an increase in reproductive tract infections and reproductive failures were observed (Coppock, Mostrom, Sparling, Jacobsen, & Ross, 1990).

Apart from causing health problems in humans and animals, mycotoxins are also responsible for economic losses occurring at all levels of food and feed production. Prior to 2012, Brazilian regulations were restricted to aflatoxin contamination in peanuts intended for human consumption, and maize destined for animal feed. However, since 2012, the Brazilian Health Surveillance Agency (ANVISA) has proposed a maximum tolerated limit (MTL) of fumonisins, zearalenone, ochratoxin and deoxynivalenol in maize and maize-based products (Brasil, 2011), to be fully implemented by 2016.

To date, most Brazilian studies have dealt with single mycotoxins or mycotoxin classes, thus only dealing with a limited number of chemically related target analytes. Considering that more than one mycotoxin can occur naturally and new MTLs have been set by ANVISA, it is important to perform broad and reliable analyses to identify which fungal metabolites are co-occurring in maize samples. Therefore, the aim of this work was to determine the identities and levels of fungal and microbial metabolites contaminating maize grains from farmers' sites in the South region of Brazil.

2. Material and Methods

2.1. Samples

A total of one hundred and forty-eight maize samples were collected from farmers' sites in Rio Grande do Sul (n=51), Santa Catarina (n=40) and Paraná (n=57) states, from 2012 to 2013. Samples (1 kg each) were properly labelled with sample location and collection date. Each whole sample was ground through a 2.0 mm sieve (ZM 200; Retsch, Haan, Germany) and partitioned using a sample divider (PT 100; Retsch).

2.2. Mycotoxin analysis

2.2.1. Chemicals and reagents

Methanol (LC gradient grade) and glacial acetic acid (p.a.) were purchased from Merck (Darmstadt, Germany); acetonitrile (LC gradient grade) from VWR (Leuven, Belgium); and ammonium acetate (MS grade) from Sigma-Aldrich (Vienna, Austria). Standards for mycotoxins were obtained from various research groups or from commercial sources. Water was purified successively by reverse osmosis with an Elga Purelab ultra analytic system from Veolia Water (Bucks, UK).

2.2.2. Maize sample extraction

Twenty milliliters of extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) was added to five grams of maize sample weighed in a 50-ml polypropylene tube. Samples were extracted for 90 min on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany). After sedimentation by gravity, each sample was diluted in a 1:1 ratio with dilution solvent (acetonitrile/water/acetic acid 20:79:1, v/v/v) and 5 µl of the diluted extract was injected into an LC-MS/MS system (Sulyok, Berthiller, Krska, & Schuhmacher, 2006). For spiking experiments, 0.25 g of three individual samples, considered to lack fungal and bacterial metabolites, were weighed and spiked at one concentration level. The spiked samples were mixed and stored at room temperature overnight in the dark to establish equilibrium between the analytes and the sample.

2.2.3. LC-MS/MS parameters

Maize analyses were performed by the multi-mycotoxin LC–MS/MS method developed by Sulyok, Berthiller, Krska, and Schuhmacher (2006), which was recently updated and currently covers 295 different mycotoxins and other microbial metabolites (Malachova et al., 2014). Briefly, analyses were performed with a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray electrospray ionisation (ESI) source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was undertaken at 25 ˚C on a Gemini C_{18}-column, 150 x 4.6 mm i.d., 5 µm particle size, equipped with a C_{18} 4 x 3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA).

ESI-MS/MS was performed in the time-scheduled multiple reaction monitoring (MRM) mode, both in positive and negative polarities, in two separate chromatographic runs per sample, by scanning two fragmentation reactions per analyte. The MRM detection

window of each analyte was set to its expected retention time ±27 s and ±48 s in the positive and negative mode, respectively. Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte (with the exception of moniliformin and 3-nitropropionic acid that exhibit only one fragment ion). This yielded 4.0 identification points according to European Union Commission decision 2002/657 (EC, 2002). In addition, the LC retention time and the intensity ratio of the two MRM transitions agreed with the related values of an authentic standard within 0.1 min and 30% relative, respectively.

Quantification was performed using an external calibration based on serial dilutions of a multi-analyte stock solution. As no quantitative standard was available for norsolorinic acid (NOR), averufin (AVER), averufanin (AVFN), versicolorins A (VER-A) and C (VER-C), fumonisins B_4 (FB_4) and B_6 (FB_6), these compounds were semi-quantified using the response factor of the structurally related compound averantin (for NOR, AVER, AVFN, VER-A and VER-C) and fumonisins B_2 (FB_2) and B_1 (FB_1), respectively. Results were corrected for apparent recoveries obtained during in-house validation of the method for maize (Malachova et al. 2014).

3. Results and Discussion

3.1. Regulated mycotoxins and correlated metabolites

A total of seventy-seven metabolites were detected from maize samples, of which at least two are addressed by Brazilian legislation. Table 1 shows the results of regulated mycotoxin contamination classified by state.

We found fumonisin B_1 and fumonisin B_2 to be present in all maize samples while the highest levels of FB_1 + FB_2 were detected in a sample from Paraná state. Fumonisin B_3, fumonisin B_4 and fumonisin B_6 were also detected in 98.6, 97.9 and 8.1% of maize samples, respectively. Previous studies in Brazil also reported high frequencies and levels of fumonisin contamination: Van der Westhuizen, et al., (2003) analysed 90 corn samples from Santa Catarina, finding fumonisin B_1, B_2 and B_3 in all samples, with total fumonisin levels ranging from 20.0 to 18,740 µg/kg. Ono, et al., (2001) found contamination by fumonisins in 98% of maize samples, with levels ranging from 960.2 to 22,600 µg/kg. In maize samples analysed by Queiroz, et al., (2012), all samples from Minas Gerais, Brazil were contaminated by fumonisins (FB_1 + FB_2) in the range from 230.1 to 6,450 µg/kg. In this current study, hydrolysed fumonisin B_1 (HFB_1), the major hydrolysis product of FB_1 formed during alkaline

treatment of maize, was detected in 38% of non-processed maize samples. Our results showing high levels of fumonisin contamination thus match previous studies.

Zearalenone (ZON), being the second most commonly regulated mycotoxin detected, was present in 73.6% of maize samples. The highest contamination of ZON in a single maize sample (5,088 µg/kg), was detected in a sample from Rio Grande do Sul state. Similarly, Queiroz, et al., (2012) reported the presence of ZON in 95% of maize samples from Brazil, with levels from 1.8 to 99.0 µg/kg; the present study, in comparison, indicated the frequency of positive samples to be lower but the levels of ZON to be higher. A similar frequency of positive samples was demonstrated by Oliveira, et al., (2009), which analysed twenty hybrids of maize from the South region of Brazil: ZON was found in 75% of samples, in concentrations varying from 50 to 640 µg/kg; zearalenone-4-sulphate and *beta*-zearalenol, two derivatives of ZON, were also found in 41 and 31% of samples, at a maximum level of 104.4 and 222.1 µg/kg, respectively.

Aflatoxins B_1 and G_1 were present in maize samples from all states. The highest contaminated sample was from Santa Catarina (49.9 µg/kg), however the frequency was lower than for Paraná or Rio Grande do Sul (Table 1). The current results are in agreement with earlier studies performed in maize and maize-based products from Brazil, where a low frequency of aflatoxin contamination was detected (Amaral, Nascimento, Sekiyama, Janeiro, & Machinski Jrs, 2006; Souza, et al., 2013). However the present study found aflatoxin contamination at levels above the maximum tolerated limit (MTL). This finding is important since maize crops without aflatoxin contamination sometimes become combined with maize highly contaminated by aflatoxins, producing a final product that is unlawful (Carlson & Ensley, 2003). Additionally, aflatoxin M_1 was found in four maize samples while aflatoxin B_2 and aflatoxin G_2 were not detected.

The Brazilian MTL for deoxynivalenol (DON) in maize, is not due to be applied until 2016. DON was detected in seventy-one maize samples (48%); however no sample demonstrated DON contamination higher than the MTL. However, the frequency of DON contamination found in this present study differs from previous studies conducted in Brazil. For instance, Souza, et al. (2013) reported a frequency of DON contamination in 4% of maize samples, with a maximum level of 30 µg/kg. Low rates of contamination and levels of DON were also found by Milanez, Valente-Soares, and Baptista (2006); the DON derivatives, 3-acetyldeoxynivalenol (3AcDON) and 15-acetyldeoxynivalenol (15AcDON) were present in 5.3 % and 49.9 % of maize samples, respectively. Contamination by these derivatives contributed, on average, to 4.2 % and 31% of the sum of DON and it derivatives' total

contamination, it being an average percentage contribution of 15AcDON higher than that previously reported (EFSA, 2013). Deoxynivalenol-glucoside, a glucoside derivative of DON, which occurs naturally in *Fusarium*-contaminated maize, was found in 15% of maize samples, with a mean of 2.6 µg/kg and a maximum contamination level of 115.1 µg/kg.

3.2. Co-contamination by regulated mycotoxins

Table 2 summarises the number of samples by state and the frequency of co-occurrence of regulated mycotoxins. Since FB_1 and FB_2 were present in all samples, it was expected that combinations with fumonisins would be the most commonly found as shown in Table 2. This result differs from the research of others showing a low co-occurrence between fumonisins and ZON. For instance, Garrido, Hernández Pezzani, & Pacin, (2012) found a 2.03% frequency of co-occurrence between FB_1 + FB_2 and ZON in Argentinian maize from 1999 to 2010. Kawashima & Valente-Soares, (2006) reported a high occurrence of fumonisins in corn products (94%); however ZON was not found in samples from Brazil.

The current results indicate that the frequency of co-occurrence of aflatoxins and fumonisins in maize is lower than previous studies performed in Brazil. On the other hand, this study found some aflatoxin levels in individual samples were above the Brazilian MTL. Cruz, (2010) reported a frequency of aflatoxins (AFB_1 + AFB_2 + AFG_1 + AFG_2) and FB_1 + FB_2 in 41.66% and 83.3%, respectively, of maize samples; however all aflatoxin levels were below the MTL. Oliveira, et al. (2006) found 64.5% co-occurrence of AFB_1 and FB_1 in poultry feed samples.

3.3. Non-regulated metabolites by Brazilian legislation

Apart from non-regulated metabolites occurring in maize samples, we found other metabolites from *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and also bacterial species (Table 3).

Among the *Aspergillus* metabolites, 3-nitropropionic acid (3-NPA) and averufin (AVE) were detected in 83 (56.8%) and 46 (31.1%) of total maize samples, respectively. The maximum concentration of 3-NPA measured in this current study was higher than that detected by Ezekiel, Sulyok, Warth, Odebode, & Krska (2012) in peanut cake, and Ezekiel, Bandyopadhyay, Sulyok, Warth, & Krska (2012) in poultry feed samples in Nigeria. Other *Aspergillus* metabolites found in relatively few samples were versicolorin-A (VER-A),

versicolorin-C (VER-C), nidurufin (NID) and sterigmatocystin (STE), methylsterigmatocystin and methoxysterigmatocystin. As with averufin, all of these metabolites are involved in the aflatoxin B_1 biosynthetic pathway (Ezekiel et al., 2012), which is classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC, 2002). Kojic acid, also an *Aspergillus* metabolite, was detected in 5.4% of maize samples, reaching the maximum concentration of 1,719 µg/kg. This result differs from that of Souza et al. (2013), who reported that 100% of maize and poultry feed was contaminated with kojic acid, with maximum levels of 344 µg/kg and 28 µg/kg, respectively.

Non-regulated metabolites from *Fusarium* species, namely beauvericin (BEA), aurofusarin (AUR), nivalenol (NIV), moniliformin (MON), fusaric acid (FUA) and fusarin C (FUC), were found in at least 50% of samples. Our results are similar to those of Souza et al. (2013), who reported the presence of BEA in 96% of maize samples, ranging in concentrations from 12 to 160 µg/kg. AUR was found in 128 maize samples (86.5%) with a mean concentration of 1,430 µg/kg and a maximum of 20,448 µg/kg. The level of AUR contamination was higher than that found by Chala et al. (2014), who found AUR in sorghum and finger millet at a maximum concentration of 1,108 µg/kg. NIV was found in 112 samples (75.6%), with a mean concentration of 256.2 µg/kg and maximum of 3,646 µg/kg. As well as DON, NIV was classified by IARC as Group 3 – not classifiable relating to its carcinogenicity to humans (IARC, 1993); however only DON had an MTL set by Brazilian regulation. The frequency and maximum level of NIV contamination demonstrated by our study was higher than that found for DON, necessitating additional discussion about thrichotecenes contamination. MON was present in 72.9% of samples, with the highest level found being 645.8 µg/kg, a level 3.5 times higher than that reported by Souza et al. (2013). FUA was found at a lower frequency than that reported by Abia et al. (2013); however the mean concentration (419.8 µg/kg) was nine times higher than that previously reported and the maximum level detected was more than ten times higher. Even though it is classified by IARC in 2B group, even as FB_1 and FB_2, Fusarin C (FUC), is not included in any legislation. Samples from SC showed a high frequency 65% of FUC, followed by PR and RS, which shown a frequency of 52,6 and 45,1%, respectively. FUC occured in samples from SC in higher frequency than other states, maximum level of FUC detected in samples from SC was 195.3 µg/kg, it was lower than 1,177 and 1,476 µg/kg. Levels found in RS an PR, respectively. FUC is instable to light and under extraction conditions and rearrangement to various isomers can occur, may result in underestimates of amount of fusarin presente in maize samples (Kleigrewe, Sohnel & Humpf, 2011; Kleigrewe, et al., 2012). Although,

Savard & Miller (1992) reported that mutagenicity of extracts containing FUC is greater than can be explained on the basis of measured FUC. Few information about production under field conditions are available. Kleigrewe, et al. (2011) developed a HPLC method to determine fusarin C in maize and maize-based products. Levels of fusarin C varid in maizes and maize-based products from not detectable up to 83 mg/kg and in food samples from not detectable up to 28 µg/kg.

Other *Fusarium* metabolites detected were diacetoxyscirpenol, monoacetoxyscirpenol, enniatin A, A_1, B, B_1, culmorin, 15-hydroxyculmorin, 5-hydroxyculmorin, 15-hydroxyculmoron, equisetin, fusapyron, deoxyfusapyron, bikaverin, monocerin, apicidin, rubrofusarin, chrysogine, fusaproliferin and decalonectrin.

Penicillium metabolites, including mycophenolic acid, andrastin A, curvularin and penicillic acid were also detected, although the prevalence of individual metabolites was not high. Additionally, metabolites from other fungal species (brevinamid F, cytochalasin J, cytochalasin E, cladosporin, pestalotin, radicicol, brefeldin A and cycloaspeptide A) and unspecified metabolites (rugulusovine, tryptophol, emodin, citrinin, rugulosin, butenolid, calphostin C and chanoclavin) were recovered from maize samples.

Alternaria metabolites (alternariol, alternariol-methyl-ether, macrosporin A and tentoxin) were found in maize samples, but at low frequency and contamination levels.

Nonactin and monactin, both bacterial metabolites, were also detected in our maize samples.

4. Conclusions

Fungi and other bacteria contaminants in maize producing mycotoxins and bacterial metabolites toxic to humans and animals is a major problem. Fumonisins were detected in all maize samples at high levels, and fusarin C detected in more than fifty percent of samples, demonstrate these posed the biggest threat. Considering the potential risks associated with each mycotoxin alone, the co-occurrence of mycotoxins could increase the likelihood of human and animal morbidity and mortality. The identification of various mycotoxin combinations would be useful in estimating any possible synergistic or additive effects in contaminated food or feed. Future studies are required to increase our knowledge of the local co-occurrences of mycotoxins, with the aim of reducing maize impacts.

Acknowledgements

This work was carried out during a sandwich internship within the PhD programme of the first author. We would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível de Superior (CAPES), for granting a scholarship to the first author. The LC-MS/MS system was funded by the Federal Country Lower Austria and co-financed by the European regional development fund of the European Union.

References

AGEITEC, (n.d.) Agência Embrapa de Informação Tecnológica. *Importância Socioeconômica do milho*. Retrieved June 19, 2014, from http://www.agencia.cnptia.embrapa.br/gestor/milho/arvore/CONTAG01_8_168200511157.html

Abia, W. A., Warth, B., Sulyok, M., Krska, R., Tchana, A. N., Njobeh, P. B., Dutton, M. F., & Moundipa, P. F. (2013). Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). *Food Control, 31*(2), 438-453.

Amaral, K. A. S. d., Nascimento, G. B., Sekiyama, B. L., Janeiro, V., & Machinski Jrs, M. (2006). Aflatoxinas em produtos à base de milho comercializados no Brasil e riscos para a saúde humana. *Food Science and Technology (Campinas), 26*, 336-342.

Bracarense, A. P. F. L., Lucioli, J., Grenier, B., Drociunas, P. G., Moll, W. D., Schatzmayr, G., & Oswald, I. P. (2012). Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets. *British Journal of Nutrition, 107*(12), 1776-1786.

Brasil, 2011. Agência Nacional de Vigilância Sanitária (ANVISA). Resolução RDC No 7, de 18 de fevereiro de 2011. Dispõe sobre limites máximos tolerados (LMT) para micotoxinas em alimentos. Diário Oficial da União – Ministério da Saúde, Brasília, DF, Brasil, de 22/03/2011. Retrieved from: http://portal.anvisa.gov.br/wps/wcm/connect/bc17db804f45fe2cbd41fdd785749fbd/Resolu%C3%A7%C3%A3o+0-2011-CGALI.pdf?MOD=AJPERES

Carlson, M. P., & Ensley, S. M. (2003). Use of Feed Contaminated with Fungal (Mold) Toxins (Mycotoxins). *Plant Diseases, Corn*(June).

Chala, A., Taye, W., Ayalew, A., Krska, R., Sulyok, M., & Logrieco, A. (2014). Multimycotoxin analysis of sorghum (Sorghum bicolor L. Moench) and finger millet (*Eleusine coracana* L. Garten) from Ethiopia. *Food Control, 45*(0), 29-35.

CONAB, (2014). Companhia Nacional de Abastecimento. *Séries históricas – Milho 1º e 2º* safra. Retrieved June 10, 2014, from http://www.conab.gov.br/conteudos.php?a=1252&ordem=produto&Pagina_objcmsconteudos=3#A_objcmsconteudos

Coppock, R. W., Mostrom, M. S., Sparling, C. G., Jacobsen, B., & Ross, S. C. (1990). Apparent zearalenone intoxication in a dairy herd from feeding spoiled acid-treated corn *Veterinary and Human Toxicolology, 32*, 246-248.

Cortinovis, C., Caloni, F., Schreiber, N. B., & Spicer, L. J. (2014). Effects of fumonisin B1 alone and combined with deoxynivalenol or zearalenone on porcine granulosa cell proliferation and steroid production. *Theriogenology, 81*(8), 1042-1049.

Cruz, J. V. da S. (2010). Ocorrência de aflatoxinas e fumonisinas em produtos à base de milho e milho utilizado como ingrediente de ração para animais de companhia, comercializados na região de Pirassununga. Estado de São Paulo [*PhD thesis*]. Pirassununga (SP): São Paulo University.

EFSA. (2013). Deoxynivalenol in food and feed: occurrence and exposure. *European Food Safety Authority Journal, 11(10)*(3379), 56.

EC, 2002. (EC) European Commission. Commission Decision (EC) No 657/2002 of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of European Communities (2002), pp. L221–L236

Ezekiel, C. N., Bandyopadhyay, R., Sulyok, M., Warth, B., & Krska, R. (2012). Fungal and bacterial metabolites in commercial poultry feed from Nigeria. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment, 29*(8), 1288-1299.

Ezekiel, C. N., Sulyok, M., Warth, B., Odebode, A. C., & Krska, R. (2012). Natural occurrence of mycotoxins in peanut cake from Nigeria. *Food Control, 27*(2), 338-342.

Farias, A. X. D., Robbs, C. F., Bittencourt, A. M., Andersen, P. M., & Corrêa, T. B. S. (2000). Contaminação endógena por Aspergillus spp. em milho pós-colheita no Estado do Paraná. *Pesquisa Agropecuária Brasileira, 35*, 617-621.

Garrido, C. E., Hernández Pezzani, C., & Pacin, A. (2012). Mycotoxins occurrence in Argentina's maize (Zea mays L.), from 1999 to 2010. *Food Control, 25*(2), 660-665.

Grenier, B. & Oswald, I. P. (2011). Mycotoxin co-contamination of food and feed: meta-analysis of publications describing toxicological interactions. *World Mycotoxin Journal, (4)*285-313.

IARC. (1993). Toxins derived from *Fusarium graminearum, F. culmorun* and *F. crookwellense*: zearalenone, deoxynivalenol, nivalenol and fusarenone x (Group 3). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 56,* 397.

IARC. (2002). Traditional herbal medicines, some mycotoxins, naphtalene, and styrene. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 82,* 169-366.

Kawashima, L. M., & Valente Soares, L. M. (2006). Incidência de fumonisina B1, aflatoxinas B1, B2, G1 e G2, ocratoxina A e zearalenona em produtos de milho. *Food Science and Technology (Campinas), 26,* 516-521.

Kleigrene, K., Sohnel, A-C., & Humpf, H-U. (2011). A New High-Performance Liquid Chromatography–Tandem Mass Spectrometry Method Based on Dispersive Solid Phase Extraction for the Determination of the Mycotoxin Fusarin C in Corn Ears and Processed Corn Samples. Journal of Agricultural and Food Chemistry 2011 59 (19), 10470-10476.

Kleigrewe, K., Aydin, F., Hogrefe, K., Piecuch, P., Bergander, K., Würthwein, E-U. & Humpf, H-U. (2012). Structure Elucidation of New Fusarins Revealing Insights in the Rearrangement Mechanisms of the Fusarium Mycotoxin Fusarin C. *Journal of Agricultural and Food Chemistry 60*(21), 5497-5505.

Malachova, A, Sulyok M, Beltrán E, Berthiller F, & Krska R. (2014). Optimization and validation of a quantitative liquid chromatography – tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices. J. Chromatogr. A, in press, http://dx.doi.org/10.1016/j.chroma.2014.08.037

Milanez, T. V., Valente-Soares, L. M., & Baptista, G. G. (2006). Occurrence of trichothecene mycotoxins in Brazilian corn-based food products. *Food Control, 17*(4), 293-298.

Oliveira, G., Ribeiro, J., Fraga, M., Cavaglieri, L., Direito, G., Keller, K., Dalcero, A., & Rosa, C. (2006). Mycobiota in poultry feeds and natural occurrence of aflatoxins, fumonisins and zearalenone in the Rio de Janeiro State, Brazil. *Mycopathologia, 162*(5), 355-362.

Oliveira, T. R. d., Jaccoud-Filho, D. d. S., Henneberg, L., Michel, M. D., Demiate, I. M., Pinto, A. T. B., Machinski Junior, M., & Barana, A. C. (2009). Maize (Zea mays L.) landraces from the southern region of Brazil: contamination by *Fusarium* sp,

zearalenone, physical and mechanical characteristics of the kernels. *Brazilian Archives of Biology and Technology, 52*, 11-16.

Ono, E. Y. S., Ono, M. A., Funo, F. Y., Medina, A. E., Oliveira, T. C. R. M., Kawamura, O., Ueno, Y., & Hirooka, E. Y. (2001). Evaluation of fumonisin-aflatoxin co-occurrence in Brazilian corn hybrids by ELISA. *Food Additives and Contaminants, 18*(8), 719-729.

Queiroz, V. A. V., de Oliveira Alves, G. L., da Conceição, R. R. P., Guimarães, L. J. M., Mendes, S. M., de Aquino Ribeiro, P. E., & da Costa, R. V. (2012). Occurrence of fumonisins and zearalenone in maize stored in family farm in Minas Gerais, Brazil. *Food Control, 28*(1), 83-86.

Richard, J. L. (2007). Some major mycotoxins and their mycotoxicoses—An overview. *International Journal of Food Microbiology, 119*(1–2), 3-10.

Savard, M E. & Miller, J. D. (1992). Characterization of Fusarin F, a New Fusarin from *Fusarium moniliforme*. Journal of Natural Products. *55*(1), 64-70

Souza, M. d. L. M. d., Sulyok, M., Freitas-Silva, O., Costa, S., #xf4, Soares, n., Brabet, C., Machinski Junior, M., Sekiyama, B. L., Vargas, E. A., Krska, R., & Schuhmacher, R. (2013). Cooccurrence of Mycotoxins in Maize and Poultry Feeds from Brazil by Liquid Chromatography/Tandem Mass Spectrometry. *The Scientific World Journal, 2013*, 9.

Sulyok, M., Berthiller, F., Krska, R., & Schuhmacher, R. (2006). Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. *Rapid Communications in Mass Spectrometry, 20*(18), 2649-2659.

van der Westhuizen, L., Shephard, G. S., Scussel, V. M., Costa, L. L. F., Vismer, H. F., Rheeder, J. P., & Marasas, W. F. O. (2003). Fumonisin Contamination and *Fusarium* Incidence in Corn from Santa Catarina, Brazil. *Journal of Agricultural and Food Chemistry, 51*(18), 5574-5578.

Table 1. Frequency of regulated mycotoxin contamination in maize samples by state

Mycotoxins	Location											
	PR (n=57)				SC (n=40)				RS (n=51)			
	Mean (µg/kg)	Range (µg/kg)	Frequency (%)	Frequency (%) > MTL	Mean (µg/kg)	Range (µg/kg)	Frequency (%)	Frequency (%) > MTL	Mean (µg/kg)	Range (µg/kg)	Frequency (%)	Frequency (%) > MTL
AFLAS[a]	3.1	0.4 – 16.7	11 (19.2)	0 (0.0)	16.37	0.5 – 49.9	4 (10)	1 (2.5)	7.8	0.7 – 35.1	23 (45)	2 (3.9)
FB_1[b]+FB_2[c]	3,153	63.8 – 66,274	57 (100)	3 (5.2)	2,204	62.4 – 10,080	40 (100)	5 (12.5)	2,726	120.5 – 24,581	51 (100)	6 (11.7)
ZON[d]	92.7	1.4 – 444.5	42 (73.6)	2 (3.5)	141.3	1.5 – 729.2	26 (65)	3 (7.5)	191.5	1.4 – 5,088	41 (80.4)	2 (3.9)
DON[e]	184.4	41.6 – 1,008	33 (58)	0 (0.0)	138.3	41.7 – 372.4	9 (22.5)	0 (0.0)	126.4	35.6 – 447.9	29 (56.8)	0 (0.0)

PR = Paraná; SC = Santa Catarina; RS = Rio Grande do Sul. MTL = Maximum tolerated limit; n = number of samples
[a] AFLAS = sum of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2; MTL = 20 µg/kg
[b] FB_1 = fumonisin B_1; [c] FB_2 = fumonisin B_2; MTL = 5,000 µg/kg
[d] ZON = zearalenone; MTL = 400 µg/kg
[e] DON = deoxynivalenol; MTL = 3,000 µg/kg

Table 2. Natural co-contaminating, regulated mycotoxins in maize samples

Mycotoxins	Location			Frequency of co-occurrence (%)
	PR (n=57)	SC (n=40)	RS (n=51)	
FB_1[b]+FB_2[c] & ZON[d]	42	26	41	109 (73.6)
FB_1+FB_2 & DON	33	9	29	71 (47.9)
ZON & DON	24	4	25	53 (35.8)
AFLAS[a] & FB_1+FB_2	11	4	23	38 (25.6)
AFLAS & ZON	9	2	19	30 (20.2)
AFLAS & DON	8	2	11	21 (14.2)
AFLAS & ZON & DON	6	1	11	18 (12.1)

PR = Paraná; SC = Santa Catarina; RS = Rio Grande do Sul; n = total number of samples
[a] AFLAS = sum of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2;
[b] FB_1 = fumonisin B_1; [c] FB_2 = fumonisin B_2;
[d] ZON = zearalenone;
[e] DON = deoxynivalenol.

Table 3. Natural co-contaminating fungal and bacterial non-regulated metabolites in maize from the South region of Brazil

Metabolites	Frequency (%)	Mean (µg/kg)	Maximum concentration (µg/kg)
Fusaric acid	94 (63.5)	419.8	17,164
Brevinamid F	85 (57.4)	1.51	26.4
Culmorin	84 (56.7)	114.9	2,492
3-Nitropropionic acid	83 (56.8)	120.1	1,544
Rugulosin	83 (56.8)	120.1	1,544
Kojic acid	8 (5.4)	58.9	1,719
Enniatin B_1	8 (5.4)	0.02	1.3
Curvularin	8 (5.4)	0.35	38.4
Emodin	8 (5.4)	2.63	180.5
Citrinin	8 (5.4)	12.2	1,54
Fusarin C	79 (53.3)	55.7	1,476
Rubrofusarin	70 (47.3)	516.3	7,713
Sterigmatocystin	7 (4.7)	0.08	3.3
Versicolorin C	7 (4.7)	5.14	288.2
Equisetin	65 (43.9)	7.91	237.1
15-Hydroxyculmorin	63 (42.5)	151.3	2,961
Monoacetoxyscirpenol	6 (4)	0.97	91.6
Apicidin	6 (4)	0.06	4.2
Alternariolmethylether	6 (4)	0.13	7.9
Macrosporin A	6 (4)	0.21	12.9
Andrastin A	6 (4)	3.72	429.5
Cytochalasin J	6 (4)	0.42	29.8
Cytochalasin E	6 (4)	0.09	4.3
Fusaproliferin	58 (39.1)	158.8	2,925
15-Hydroxiculmoron	52 (35.1)	30.4	476.9
Alternariol	5 (3.3)	0.16	9.4
Cycloaspeptide A	5 (3.3)	1.46	181.3
Nonactin	5 (3.3)	0.08	10.9
Averufin	46 (31.1)	47.5	3,876
Brefeldin A	44 (29.7)	3.74	48.4
Fusapyron	41(27.7)	9.34	236.9
Pestalotin	38 (25.6)	9.21	217.1
Cladosporin	38 (25.6)	4.82	188.9
5-Hydroxyculmorin	30 (20.2)	8.09	151.7
Nidurufin	3 (2)	0.25	12.1
Methylsterigmatocystin	3 (2)	0.51	37.1
Methoxysterigmatocystin	3 (2)	0.031	2.4
Chrysogine	3 (2)	0.21	11.3
Monactin	3 (2.1)	0.25	36.7
Butenolid	29 (19.6)	0.44	4.45
Rugulusovine	28 (18.9)	1.55	54.4
Radicicol	28 (18.9)	0.16	7.1
Enniatin B	27 (18.2)	0.02	0.7
Penicillic Acid	23 (15.5)	0.84	11.4
Decalonectrin	2 (1.3)	0.33	36.9
Diacetoxyscirpenol	19 (12.8)	0.59	9.9
Deoxyfusapyron	19 (12.8)	2.23	194.2
Monocerin	19 (12.8)	0.34	8.5
Chanoclavin	19 (12.8)	9.16	164.9
Tryptophol	19 (12.8)	7.84	141.1
Beauvericin	146 (98.6)	10.7	195.5
Bikaverin	138 (93.2)	75.5	835.9

Table 3. Continued.

Metabolites	Frequency (%)	Mean (µg/kg)	Maximum concentration (µg/kg)
Aurofusarin	128 (86.5)	1,43	20,448
Nivalenol	112 (75.6)	256.2	3,646
Mycophenolic acid	11 (7.4)	11.23	965.1
Moniliformin	108 (72.9)	61.7	645.8

ARTIGO 2

Free and hidden fumonisins in Brazilian raw maize samples

Maurício S. Oliveira[a], Andressa C. L. Diel[a], Ricardo H. Rauber[b], Fabiana P. Fontoura[a], Adriano Mallmann[a], Paulo Dilkin[a], Carlos A. Mallmann[a] *

(Artigo submetido ao periódico *Food Control* – 2014)

[a] Universidade Federal de Santa Maria (UFSM), Laboratório de Análises Micotoxicológicas (LAMIC), CEP 97105 900, Santa Maria, Rio Grande do Sul, Brasil

[b] Programa de Pós-graduação em Zootecnia – Universidade Tecnológica Federal do Paraná (UTFPR) – Bolsista de Pós-doutorado em empresa Fundação Araucária e Coordenação de Aperfeiçoamento de Pessoal de Nível de Superior (CAPES) - CEP 80610 190, Curitiba, Paraná, Brasil

* Corresponding author. Tel.: +55 55 3220 8445

E-mail addresses: mallmann@lamic.ufsm.br (Carlos A. Mallmann)

Free and hidden fumonisins in Brazilian raw maize samples

Maurício S. Oliveira[a], Andressa C. L. Diel[a], Ricardo H. Rauber[b], Fabiana P. Fontoura[a], Adriano Mallmann[a], Paulo Dilkin[a], Carlos A. Mallmann[a] *

[a] Universidade Federal de Santa Maria (UFSM), Laboratório de Análises Micotoxicológicas (LAMIC), CEP 97105 900, Santa Maria, Rio Grande do Sul, Brasil

[b] Programa de Pós-graduação em Zootecnia – Universidade Tecnológica Federal do Paraná (UTFPR) – Bolsista de Pós-doutorado em empresa Fundação Araucária e Coordenação de Aperfeiçoamento de Pessoal de Nível de Superior (CAPES) - CEP 80610 190, Curitiba, Paraná, Brasil

* Corresponding author. Tel.: +55 55 3220 8445

E-mail addresses: mallmann@lamic.ufsm.br (Carlos A. Mallmann)

Abstract

Fumonisins are secondary metabolites produced primarily by fungi strains that belong to the genera *Fusarium* and *Alternaria*, which have been shown to be highly prevalent in maize crops. Recently some authors have documented the presence of hidden forms of fumonisins occurring in raw maize. This purpose of this study was to determine the occurrence of free and hidden fumonisins in raw maize. The concentrations of fumonisins in 72 naturally contaminated maize samples were analyzed using liquid chromatography coupled to mass spectrometry. We evaluated the performance parameters of this method to determine free fumonisins forms (FB_1 and FB_2) and hydrolysed fumonisins forms (HFB_1 and HFB_2) using the standards from the Commission of the European Communities (decision 2002/657/EC). The analytical methods employed fell within the established guidelines. The amount of total fumonisins measured based on the hydrolyzed forms (HFB_1 + HFB_2) was 1.5–3.8 times greater than the amount of free fumonisins (FB_1 + FB_2). We then calculated the concentration of hidden fumonisins by subtracting the levels of free fumonisins from the total fumonisin levels. The levels of hidden fumonisins were calculated to be 0.5–2.0 times greater than the level of free fumonisins. A strong positive correlation (R = 0.97) was observed between free fumonisins (FB_1 + FB_2) and. total fumonisins (HFB_1 + HFB_2). Based on this correlation, we generated a predictive model to estimate the total fumonisin level based on the measured/reported free fumonisin concentration. These results show that the risk of exposure

to fumonisins is likely underestimated if only free fumonisins are considered. However, the predictive model we propose could be a novel approach to estimating the total amount of fumonisins in maize samples without needing to perform expensive and time-consuming analytical methods.

Keywords: Mycotoxin; Masked fumonisins; Hydrolyzed fumonisins; Mass spectrometry

Highlights

- Brazilian maize samples were found to contain high levels of hidden fumonisins.
- Hidden fumonisins increased in samples highly contaminated with free fumonisins.
- Hidden fumonisins were detected in raw maize samples that lacked free fumonisins.
- Levels of total and free fumonisins showed a strong positive correlation.

Chemical Compound: Fumonisin B_1 (PubChem CID: 3431); Fumonisin B_2 (PubChem: CID 3432)

Abbreviations: FB_1, fumonisin B_1; FB_2, fumonisin B_2; HFB_1, Hydrolyzed fumonisin B_1; HFB_2, Hydrolyzed fumonisin B_2

1. Introduction

Fumonisins are secondary metabolites produced by fungi strains in the genera *Fusarium* and *Alternaria*, primarily by *F. verticillioides* and *F. proliferatum*, which infect agricultural commodities during while the plants are growing and after harvest, during storage (Berthiller, Schuhmacher, Adam, & Krska, 2009, 2013; Bezuidenhout et al., 1988). Fumonisin B_1 (FB_1), and Fumonisin B_2 (FB_2) are the most abundant fumonisins in maize and therefore, they have been the most studied (Shephard, Thiel, Stockenstrom, & Sydenham, 1996).

The chemical structure of fumonisins is characterized by a polyhydroxy alkyl amine chain, diesterified with molecules of tricarballylic acid (TCA) (Bezuidenhout et al., 1988; Dall'Asta et al., 2009a). Fumonisins are structural analogs of sphingoid bases and can effectively inhibit the ceramide synthetase enzyme involved in the biosynthesis of

sphingolipids. This disrupts the normal metabolism of these bases, which is widely recognized as the mechanism for fumonisin induced damage in animals and humans (Voss, Smith, & Haschek, 2007; Wang, Norred, Bacon, Riley, & Merrill, 1991).

FB_1 causes different toxic responses in human and animals. Studies have associated an increased risk of human esophageal cancer with the consumption of maize contaminated with FB_1 (Kim, Scott, & Lau, 2003; Rheeder et al., 1992) and DNA damage in human fibroblasts (Kim et al., 2003). Given that it likely has carcinogenic effects, the International Agency for Research on Cancer (IARC) has classified FB_1 in group 2B, as a possible carcinogenic compound for humans. FB_1 has different effects in animals, like leucoencephalomalacea in horses, pulmonary edema in swine (Haschek, Gumprecht, Smith, Tumbleson, & Constable, 2001; Voss et al., 2007), and carcinogenic effects in several animal species (Muller, Dekant, & Mally, 2012). It has also been reported to be detrimental to broiler's performance and physiological responses (Rauber et al., 2013).

Hidden fumonisins were long believed to occur only after food processing (Berthiller et al., 2009). However, recently Dall'Asta (2010) showed that hidden fumonisins can also be found in unprocessed maize. The exact chemical reactions, which are responsible for the occurrence of naturally hidden forms, are still unknown (Berthiller et al., 2009, 2013; Dall'Erta et al., 2013). Hidden fumonisins cannot be directly analyzed and have to be released from the matrix by a sample treatment that converts them into extractable forms (Berthiller, Sulyok, Krska, & Schuhmacher, 2007; Galaverna, Dall'Asta, Mangia, Dossena, & Marchelli, 2009; Seefelder, Knecht, & Humpf, 2003).

Understanding that there are hidden fumonisins in raw maize suggests that the total content of fumonisins might be underestimated if the sample is inappropriately prepared before undertaking a chromatographic analysis. Several authors have reported the use of an alkaline hydrolysis step to prepare food samples to determine the total content of fumonisins (Dall'Asta, Galaverna, Aureli, Dossena, & Marchelli, 2008; Kim et al., 2003; Park, Scott, Lau, & Lewis, 2004). The unesterified polyols obtained from FB_1 and FB_2 after the alkaline hydrolysis are called hydrolyzed FB_1 (HFB_1) and hydrolyzed FB_2 (HFB_2), respectively (Galaverna et al., 2009; Park et al., 2004). Hidden fumonisins are indirectly measured as the difference between total fumonisins estimated by the hydrolyzed forms and free forms (Park et al., 2004). In 2011, the Brazilian Health Surveillance Agency (ANVISA), proposed a maximum tolerated limit (MTL) for $FB_1 + FB_2$ of 2,500 µg/kg for maize flour, maize cream, fubá, flakes, canjica, and canjiquinha, and 2,000 µg/kg for maize starch and maize based products (Brasil, 2011). Starting in 2014, ANVISA proposed an MTL for raw maize intended

for further processing of 5,000 µg/kg. All of these regulations are recent and none of them address hidden fumonisins. The aim of this study was to determine the total concentration of fumonisins based on the levels of HFB_1 and HFB_2, free FB_1 and FB_2, and hidden fumonisins in raw maize samples. Moreover, we propose the use of a regression equation as a predictive model to estimate the concentration of total fumonisins in samples that have already been analyzed.

2. Material and methods

2.1. Chemicals and Certified Reference Material

Purified FB_1 and FB_2 standards were purchased from Sigma-Aldrich (São Paulo, Brazil). Potassium hydroxide, formic acid, and all solvents used (LC Grade), were purchased from J.T.Baker (São Paulo, Brazil). Ultra-pure water was obtained from a Milli-Q System (Millipore, USA). Maize flour contaminated with FB_1 (591 µg/kg) and FB_2 (305 µg/kg) was provided by the Food Analysis Performance Assessment Scheme (FAPAS) (Certified Reference Material (CRM) number 2287) and used to evaluate the accuracy of the FB_1 and FB_2 measured and the sum of FB_1 and FB_2. HFB_1 and HFB_2 standards were prepared by alkaline hydrolysis following the technique described by Dall'Asta et al. (2008).

2.2. Samples

Seventy-two naturally contaminated maize samples were collected from different locations in the state of Rio Grande do Sul, Brazil, between 2012 and 2013. Samples were automatically collected following the sampling protocol published by Mallmann et al. (2013). At the laboratory, samples were ground in a ZM 200 ultra-centrifugal mill (Retsch, Germany) and partitioned with a PT 100 sample divider (Retsch, Germany). The analysis of free FB_1 and FB_2, and HFB_1 and HFB_2 were performed in duplicate.

2.3. Analysis of free fumonisins (FB_1 and FB_2)

Ten grams of maize sample was ground through a 2.0 mm screen and extracted with 50 mL water/acetonitrile (1:1, v/v) for 5 minutes in a high-speed blender. The extract was

then filtered. An aliquot of 20 µL was diluted in a 1% formic acid acetonitrile/water solution (1:1, v/v) before liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

2.4. Analysis of hydrolyzed forms (HFB_1 and HFB_2) and total fumonisins (HFB_1 + HFB_2)

Ten grams of maize sample were ground through a 2.0 mm screen and submitted to alkaline hydrolysis with 100 mL of KOH 2 M at room temperature for 10 minutes in a high-speed blender. After blending, 100 mL of acetonitrile was added and stirred for 30 minutes in a shaker at 80 rpm. An aliquot of 15 mL was dried under nitrogen flow and resuspended in 1.5 mL of 1% formic acid acetonitrile/water solution (1:1, v/v). After resuspension, the sample was filtered with a 0.45 µm filter and analyzed by LC-MS/MS. The concentration of total fumonisins was determined based on the analysis of the hydrolyzed forms after alkaline hydrolysis preparation (Dall'Asta, 2008). The content of hidden fumonisins was estimated based on the difference between total and free fumonisins (Kim, 2003; Park, 2004).

2.5. Liquid chromatography-mass spectrometry method (LC-MS/MS)

Free and hydrolyzed fumonisins were determined using the HPLC Agilent 1200 (Agilent Technologies Inc., USA) equipped with an API5000 triple quadruple mass spectrometer with an electrospray source (AB Sciex, Canada). The LC column was a Zorbax® C_{18}, 150 x 4.6 mm, 5 µm (Agilent Technologies Inc., USA) column with a C_{18} pre-column cartridge, run under a flow rate of 0.8 mL/min, at a column temperature of 40°C, and an injection volume of 5 µL. A gradient elution was performed using water (eluent A) and acetonitrile (eluent B) both acidified with 1% formic acid: 0-3.5 min isocratic step 35% B; 3.5-6 min to 70% B; 6-8 min isocratic step 70% B; finally a re-equilibration step at 35% B for 2 min to return to the initial condition. The MS source dependent parameters were: curtain gas (CUR) 20 L/min; collision-activated dissociation gas (CAD) was set to medium; source temperature 650°C, dry gas 1 (GS1) 50 L/min, dry gas 2 (GS2) 45 L/min, and the spray voltage was set to 5500 V. Detection was performed in positive mode (ESI+) using a multiple reaction monitoring (MRM) mode, by monitoring two transitions for each analyte, the primary transition was used for quantification and the second transition was used for confirmation (Table 1).

2.6. Method performance parameters

Performance parameters were evaluated using the standards from the Commission of the European Communities (commission decision 2002/657/EC) (Comission, 2002). For the free forms, the amounts of spiked fumonisins in a sample were determined as described in section 2.3. For the bound forms, the spiked samples underwent the alkaline hydrolysis procedure and then the hydrolyzed forms were determined. For all of the compounds linearity and calibration curves were evaluated based on the calibration curve at six concentration levels ranging from 125-10,000 µg/kg. The method accuracy, defined as percent recovery (%), and precision, defined as percent of coefficient of variation, was evaluated using experiments to measure the recovery of fumonisins spiked into blank maize samples at three different concentrations (125; 2,000 and 10,000 µg/kg) that covered the linear range, seven samples per level, on three different days by two different analysts. Detection limits (LODs) and quantification limits (LOQs) were estimated by spiking the blank maize samples with each analyte (FB_1, FB_2, HFB_1, and HFB_2). The LOD and LOQ were calculated at a signal to noise ratio of 3:1 and 10:1 respectively. A simple correlation was used to assess the relationship between the levels of free and total fumonisins obtained from the maize samples and a predictive equation was proposed. The predictive equation was applied to results obtained from raw maize samples analyzed for free FB_1 and FB_2 over the last four years (2010–2013) at the Laboratório de Análises Micotoxicológicas located at Universidade Federal de Santa Maria (LAMIC/UFSM). The number of samples analyzed by year was 3,790, 4,965, 3,643, and 2,285 respectively.

2.7. Statistical analysis

The results of the total free fumonisins and total fumonisins after hydrolysis were compared by Tukey's test ($P \leq 0.05$) within samples. The data were also submitted to simple correlation analysis to verify the association between free and total fumonisins. A predictive equation was proposed to estimate the total amount of fumonisins based on the amount of free (FB_1+ FB_2) fumonisins. Statistical analysis was performed using the Statgraphics Centurion computer statistical program (Statgraphics Centurion 15.2.14, Manugistics Inc., Rockville, MD, USA).

3. Results and discussion

3.1. Method performance parameters

The accuracy of the technique used to measure fumonisins was evaluated using recovery experiments (purified analytes in blank maize) and the CRM sample. For the recovery experiments the intra-day precision for FB_1, FB_2, HFB_1, and HFB_2 were 98.8, 99.2, 91.2, and 93.2, respectively. According to the commission decision 2002/657/EC for experiments adding purified standards to samples at levels > 10 µg/kg, the recovery range established must be between 80% and 110%. Thus, our measured recovery rates fall into the appropriate range. We then compared the recovery of fumonisins using our methods to the established values for the reference maize sample (CRM 2287). The results for the reference maize sample were reported as *z-scores*. For the sample results to be considered in agreement with the established reference compound, the scores had to be between -2.0 and 2.0. For the reference maize sample, the *z-scores* for FB_1 (0.4), FB_2 (0.3), and FB_1 + FB_2 (0.4) were within this range. The precision of the method was evaluated using the percent coefficient of variation (CV(%)). The inter-day precision results for FB_1, FB_2, HFB_1, and HFB_2 were 1.6, 2.5, 6.4, and 7.2, respectively. The commission decision 2002/657/EC states that the CV(%) must be below the level calculated by the Horwitz equation. For the fumonisin levels used in this study, the CV(%) recommended is 21.8 (125 µg/kg), 14.4 (2,000 µg/kg), and 11.3 (10,000 µg/kg). All of the results from the recovery experiment were in accordance with this standard. The detection limits and quantification limits were 10/125, 20/125, 35/125, and 40/125 µg/kg for FB_1, FB_2, HFB_1, and HFB_2 respectively.

3.2. Analysis of free and hidden fumonisins in maize samples

In all of the samples analyzed the concentration of total fumonisins was higher than free fumonisins. The concentration of total fumonisins was calculated based on the sum of HFB_1 and HFB_2 The levels of total fumonisins (HFB_1 + HFB_2) and total free fumonisins (FB_1 + FB_2) measured in each maize sample were compared using the Tukey's test and in all cases, they were significantly different ($P \leq 0.05$). The increase in the concentration of fumonisins is likely due to the release of hidden forms during the hydrolysis procedure. Six samples (8%) were thought to be free of fumonisins; however following hydrolysis, the concentration of total fumonisins was greater than zero. This is particularly interesting because FB_1 has low bioavailability and absorption at gastrointestinal level, and this may explain the occurrence of toxic effects even when feed contaminated with low doses is consumed (Marasas, Miller,

Riley, & Visconti, 2000; Shier, 2000). The presence of hidden fumonisins, even in maize samples that lack free fumonisins, could support the hypothesis that fumonisins associated with carbohydrates and proteins (like the hidden fumonisins) can be preferentially absorbed, and then return to the free form and cause toxic effects (Dall'Asta et al., 2008).

The ranges of concentrations of total fumonisins (HFB_1 + HFB_2) found in this study were 1.5–3.8 times the concentration of free FB_1 + FB_2. Hidden fumonisins were also present in all analyzed samples at a range of concentrations between 0.5 – 2.0 times the concentration of free fumonisins. When we compared the measured amount of total free fumonisins (FB_1 + FB_2) with the MTL allowed by ANVISA (5,000 µg/kg), 19 samples (26%) exceeded the legal limit. However, if the total concentration of fumonisins, including the hidden fumonisins was considered, the number of samples that exceeded the legal limit increased to 56 samples (78%). This represented a 52% increase in the number of samples that were not recommended for consumption. The established MTL is based on concerns regarding the damage caused by mycotoxins in humans and animals. A new MTL should be considered if the presence of hidden forms of fumonisins can be estimated for regulatory purposes (Berthiller et al., 2009; Wagacha & Muthomi, 2008).

Based on the results of free fumonisins (FB_1 + FB_2) and total fumonisins (HFB_1 + HFB_2) obtained from the 72 samples in this study, a regression equation was established to predict the total concentration of fumonisins in samples based on the results on the free fumonisins analysis (Figure 1). Analysis of the free forms of FB_1 and FB_2 is routinely performed in many laboratories, it is higher throughput, cheaper, and less time consuming than analyzing the hydrolyzed forms (Dall'Asta et al., 2009b; Kim et al., 2003; Sulyok et al., 2007). Given that six samples that had no free fumonisins (FB_1 + FB_2) had measurable levels of HFB_1 and HFB_2 after the hydrolysis step, the equation was developed using the conditions that free fumonisins (FB_1 + FB_2) = 0 and total fumonisins (HFB_1 + HFB_2) $\geq$ 0. Consistent with the findings from Dall'Asta (2010), who proposed a linear correlation model to predict the total amount of fumonisins, we found a strong positive correlation between the amount of free fumonisins forms and the amount of total fumonisins (R = 0.97 and P = 0.00). The final correlation equation was: *Total fumonisins = (0.8583+0.5615*Free fumonisins)^2*.

3.3. Retrospectively estimating the total fumonisin concentration in maize samples from 2010-2013.

We applied the correlation equation to data regarding fumonisin contamination in maize stocks retrieved from the LAMIC/UFSM database. The average contamination from free fumonisins over the previous four years (2010-2013) was used to estimate the average concentration of total fumonisins contamination by year (Figure 2). In 2010, the amount of total fumonisins was estimated to be 2.4 times the amount of free fumonisins. The total level of fumonisin contamination estimated decreased in 2011 (2.02-times) and 2012 (1.94-times) compared to the concentration of free fumonisins. However, in 2013, an increase for total fumonisins from 2012 was observed from 1.94-times to 2.02-times the amount of free fumonisins. These results indicated that the occurrence of hidden fumonisins was directly proportional to the occurrence of free fumonisin.

4. Conclusion

The performance parameters of the analytical methods used to quantify FB_1, FB_2, HFB_1, and HFB_2 met the standards in commission decision 2002/657/EC. The amount of hidden fumonisins occurring in raw maize samples was strongly correlated with the amount of free fumonisins. Thus, if we only measure the level of free fumonisins, we can expect to underestimate the overall risk of fumonisin exposure. Finally, while most of the samples analyzed did not exceed the limits for fumonisins in Brazil, this did not account for the hidden forms of fumonisins that were not considered in regulatory limits.

Acknowledgements

The work was performed and financed by Laboratório de Análises Micotoxicológicas in Universidade Federal de Santa Maria (LAMIC/UFSM) as part of the doctoral thesis of the first author. Samples and database access was provided by LAMIC/UFSM. We would like to thank Coordenação de Aperfeiçoamento de Pessoal de Nível de Superior (CAPES), for granting scholarship to the first author. Also, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação Araucária for granting a Post-Doctorate scholarship to Ricardo H Rauber. The authors are grateful to BioMed Proofreading for the manuscript English review.

References

Berthiller, F., Sulyok, M., Krska, R., & Schuhmacher, R. (2007). Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. *International Journal of Food Microbiology, 119*(1-2), 33-37.

Berthiller, F., Schuhmacher, R., Adam, G., & Krska, R. (2009). Formation, determination and significance of masked and other conjugated mycotoxins. *Analytical and Bioanalytical Chemistry, 395*(5), 1243-1252.

Berthiller, F., Crews, C., Dall'Asta, C., Saeger, S. D., Haesaert, G., Karlovsky, P., et al. (2013). Masked mycotoxins: a review. *Molecular Nutrition & Food Research, 57*(1), 165-186.

Bezuidenhout, S. C., Gelderblom, W. C. A., Gorstallman, C. P., Horak, R. M., Marasas, W. F. O., Spiteller, G., et al. (1988). Structure Elucidation of the Fumonisins, Mycotoxins from *Fusarium moniliforme*. *Journal of the Chemical Society-Chemical Communications*, 743-745.

Brasil, (2011). Agência Nacional de Vigilância Sanitária (ANVISA). Resolução RDC No 7, de 18 de fevereiro de 2011. Dispõe sobre limites máximos tolerados (LMT) para micotoxinas em alimentos. *Diário Oficial da União – Ministério da Saúde*, Brasília, DF, Brasil, de 22/03/2011. Retrieved from: http://portal.anvisa.gov.br/wps/wcm/connect/bc17db804f45fe2cbd41fdd785749fbd/Resolu%C3%A7%C3%A3o+0-2011-CGALI.pdf?MOD=AJPERES

Commission of the European Communities, 2002 Commission of the European Communities Commission regulation (EC) No. 657/2002 of 12 August 2002 concerning the performance of analytical methods and the interpretation of results. *Official Journal of European Union*, L 221 (2002), pp. 8–29.

Dall'Asta, C., Galaverna, G., Aureli, G., Dossena, A., & Marchelli, R. (2008). A LC/MS/MS method for the simultaneous quantification of free and masked fumonisins in maize and maize-based products. *World Mycotoxin Journal, 1*(3), 237-246.

Dall'Asta, C., Galaverna, G., Mangia, M., Sforza, S., Dossena, A., & Marchelli, R. (2009a). Free and bound fumonisins in gluten-free food products. *Molecular Nutrition & Food Research, 53*(4), 492-499.

Dall'Asta, C., Mangia, M., Berthiller, F., Molinelli, A., Sulyok, M., Schuhmacher, R., et al. (2009b). Difficulties in fumonisin determination: the issue of hidden fumonisins. *Analytical and Bioanalytical Chemistry, 395*(5), 1335-1345.

Dall'Asta, C., Falavigna, C., Galaverna, G., Dossena, A., & Marchelli, R. (2010). In Vitro Digestion Assay for Determination of Hidden Fumonisins in Maize. *Journal of Agricultural and Food Chemistry, 58*(22), 12042-12047.

Dall'Erta, A., Cirlini, M., Dall'Asta, M., Del Rio, D., Galaverna, G., & Dall'Asta, C. (2013). Masked mycotoxins are efficiently hydrolyzed by human colonic microbiota releasing their aglycones. *Chemical Research in Toxicology, 26*(3), 305-312.

Galaverna, G., Dall'Asta, C., Mangia, M. A., Dossena, A., & Marchelli, R. (2009). Masked mycotoxins: An emerging issue for food safety. *Czech Journal of Food Sciences, 27*, S89-S92.

Haschek, W. M., Gumprecht, L. A., Smith, G., Tumbleson, M. E., & Constable, P. D. (2001). Fumonisin toxicosis in swine: An overview of porcine pulmonary edema and current perspectives. *Environmental Health Perspectives, 109*(2), 251-257.

Kim, E. K., Scott, P. M., & Lau, B. P. (2003). Hidden fumonisin in corn flakes. *Food Additives Contaminants, 20*(2), 161-169.

Mallmann, A. O., Marchioro, A., Oliveira, M. S., Minetto, L., Wovst, L. R. S., Rauber, R. H., et al. (2013). Dois planos de amostragem para análise de fumonisinas em milho. *Ciência Rural, 43*(3), 551-558.

Marasas, W. F. O., Miller, J. D., Riley, R. T., & Visconti, A. (2000). *Environmental Health Criteria 219: Fumonisin B_1*. World Health Organization, Geneva.

Muller, S., Dekant, W., & Mally, A. (2012). Fumonisin B_1 and the kidney: modes of action for renal tumor formation by fumonisin B_1 in rodents. *Food and Chemical Toxicology, 50*(10), 3833-3846.

Park, J. W., Scott, P. M., Lau, B. P., & Lewis, D. A. (2004). Analysis of heat-processed corn foods for fumonisins and bound fumonisins. *Food Additives & Contaminants, 21*(12), 1168-1178.

Rauber, R. H., Oliveira, M. S., Mallmann, A. O., Dilkin, P., Mallmann, C. A., Giacomini, L. Z., et al. (2013). Effects of fumonisin B_1 on selected biological responses and performance of broiler chickens. *Pesquisa Veterinária Brasileira, 33*(9), 1081-1086.

Rheeder, J. P., Marasas, W. F. O., Thiel, P. G., Sydenham, E. W., Shephard, G. S., & Van Schalkwyk, D. J. (1992). *Fusarium moniliforme* and Fumonisins in Corn in Relation to Human Esophageal Cancer in Transkei. *Phytopathology, 82*(3), 353-357.

Seefelder, W., Knecht, A., & Humpf, H. U. (2003). Bound fumonisin B_1: analysis of fumonisin-B1 glyco and amino acid conjugates by liquid chromatography-electrospray

ionization-tandem mass spectrometry. *Journal of Agricultural Food Chemistry, 51*(18), 5567-5573.

Shephard, G. S., Thiel, P. G., Stockenstrom, S., & Sydenham, E. W. (1996). Worldwide survey of fumonisin contamination of corn and corn-based products. *Journal of AOAC International, 79*(3), 671-687.

Shier, W. T. (2000). The fumonisin paradox: A review of research on oral bioavailability of fumonisin B(1), a mycotoxin produced by *Fusarium moniliforme*. *Journal of Toxicology-Toxin Reviews, 19*(2), 161-187.

Sulyok, M., Krska, R., & Schuhmacher, R. (2007). A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples. *Analytical and Bioanalytical Chemistry, 389*(5), 1505-1523.

Voss, K. A., Smith, G. W., & Haschek, W. M. (2007). Fumonisins: toxicokinectics, mechanism of action and toxicity. *Animal Feed Science and Technology, 137*(3-4), 299-325.

Wagacha, J. M., & Muthomi, J. W. (2008). Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology, 124*(1), 1-12.

Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., & Merrill, A. H. (1991). Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with *Fusarium moniliforme*. *Journal of Biological Chemistry, 266*, 14486-14490.

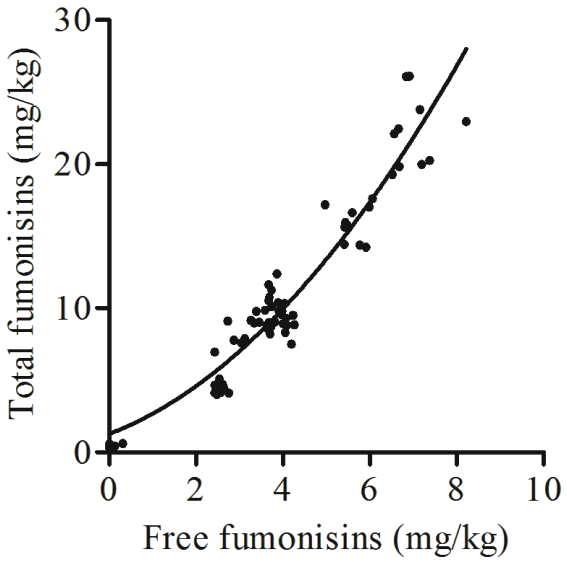

Figure 1. Correlation between free and total fumonisins concentration based on determination of hydrolyzed forms in raw maize samples. The found equation for this correlation was: *Total fumonisins = (0.8583+0.5615*Free fumonisins)^2*; R = 0.97.

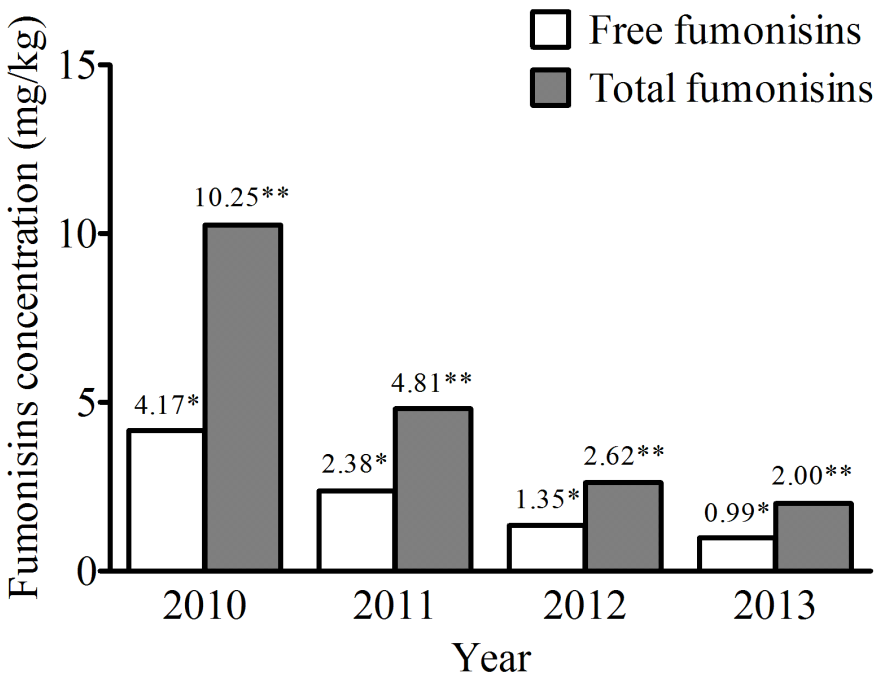

Figure 2. Relation between contamination of free fumonisins and total fumonisins in maize samples analyzed by LAMIC/UFSM. * Average of contamination of free fumonisins (FB_1 + FB_2) ** Total fumonisins concentration estimated by predictive model.

Table 1. Retention time and compound dependent parameters for LC-ESI-MS/MS analysis of free and hydrolyzed fumonisins

Compound	Retention time (min)	Precursor ion (*m/z*) $[M+H]^+$	Declustering Potential (V)	Product ions (*m/z*)[a]	Collision energy (V)[a]	Cell Exit Potential (V)[a]
FB_1	3.5	722.5	160	334.4/704.4	57/43	18/20
FB_2	7.1	706.5	160	336.4/688.4	51/41	18/20
HFB_1	2.7	406.5	140	334.5/370.5	33/30	21/21
HFB_2	6.8	390.5	140	318.5/354.5	32/26	25/28

[a] Numerical values are given in the order quantifier / qualifier ion

ARTIGO 3

Efeitos de micotoxinas do *Fusarium spp.* no desempenho de frangos de corte

Maurício S. Oliveira[1], André L. Mallmann[2], Luis A. de Cezaro[2], Diego Sturza[2], Leandro Z. Giacomini[2], Paulo Dilkin[1], Carlos A. Mallmann[1]*

(Artigo à ser submetido ao periódico *Pesquisa Veterinária Brasileira*– 2014)

[1] Universidade Federal de Santa Maria (UFSM), Laboratório de Análises Micotoxicológicas (LAMIC), CEP 97105 900, Santa Maria, Rio Grande do Sul, Brasil

[2] Instituto SAMITEC, Cx. Postal 5072, Santa Maria, RS 97105-970

* Corresponding author. Tel.: +55 55 3220 8445

E-mail address: mallmann@lamic.ufsm.br (Carlos A. Mallmann)

Efeitos de micotoxinas do *Fusarium* spp. **no desempenho de frangos de corte**

Maurício S. Oliveira[1], André L. Mallmann[2], Luis A. de Cezaro[2], Diego Sturza[2], Leandro Z. Giacomini[2], Paulo Dilkin[1], Carlos A. Mallmann[1]*

[1] Universidade Federal de Santa Maria (UFSM), Laboratório de Análises Micotoxicológicas (LAMIC), CEP 97105 900, Santa Maria, Rio Grande do Sul, Brasil

[2] Instituto SAMITEC, Cx. Postal 5072, Santa Maria, RS 97105-970

* Corresponding author. Tel.: +55 55 3220 8445

E-mail address: mallmann@lamic.ufsm.br (Carlos A. Mallmann)

ABSTRACT.- Oliveira M.S., Mallmann A.L., de Cezaro L.A., Sturza D., Giacomini L.Z., Dilkin P. & Mallmann C.A. 2013 **[Effects of *Fusarium* spp. mycotoxins on performance of broilers chickens.]**

The objective of this study was to determine the performance of broilers chickens fed with diets containing fungal culture materials produced from four different toxigenic strains of fungi of the genus *Fusarium*, isolated from clinical suspects of fusarium toxicosis concerning performance issues. Fumonisins B_1, B_2, B_3 and fusaric acid were detected in fungal culture materials. Each diet was formulated to reach 100 mg/kg of fumonisin B_1 was provided for 21 days. The concentrations of the other mycotoxins have been calculated from the amount of fungal culture embedded in each diet material. The performance parameters evaluated were: body weight, feed intake and feed conversion. Furthermore, we analyzed the sphinganine/sphingosine ratio (SA/SO) and the concentration of total plasma proteins (TPP) in the serum of broilers chickens. Body weight and feed conversion were significantly altered at 21 days in the group receiving diet containing higher concentration of fusaric acid. The ratio SA/SO and TPP levels were significantly altered at 14 and 21 days, respectively in T3 group. These results indicate that 100 mg/kg of fumonisin B_1 in the presence of fusaric acid at levels above 80 mg/kg significantly affect the performance of broilers chickens.

INDEX TERMS: Fumonisins, fusaric acid, performance, broiler chickens

RESUMO.- [**Efeitos de micotoxinas do *Fusarium* spp. no desempenho de frangos de corte.**] O objetivo deste trabalho foi avaliar o desempenho de frangos de corte submetidos a dietas contendo materiais de cultivo fúngico produzidos a partir de quatro diferentes cepas fúngicas toxígenas do gênero *Fusarium*, isoladas de casos clínicos suspeitos de

fusariotoxicose, relativo a problemas de desempenho. Diferentes concentrações de fumonisinas B_1, B_2, B_3 e ácido fusárico foram detectadas nos materiais de cultivo fúngico. Cada dieta foi formulada para conter 100 mg/kg de fumonisina B_1 foi fornecida por 21 dias. Os parâmetros de desempenho avaliados foram peso corporal, consumo de ração e conversão alimentar. Além disso, foram analisadas a relação esfinganina/esfingosina (SA/SO) e a concentração de proteínas plasmáticas totais (PPT) no soro das aves. Peso corporal e conversão alimentar foram significativamente alterados aos 21 dias no grupo que recebeu dieta contendo maior concentração de ácido fusárico. A relação SA/SO e os níveis de PPT foram significativamente alterados aos 14 e 21 dias, respectivamente no grupo T3. Estes resultados indicam que 100 mg/kg de fumonisina B_1 em presença de ácido fusárico em níveis acima de 80 mg/kg afetam significativamente o desempenho de frangos de corte.

TERMOS DE INDEXAÇÃO: Fumonisinas, ácido fusárico, desempenho, frangos de corte

INTRODUÇÃO

O gênero *Fusarium* apresenta ocorrência ubiquitária em culturas agrícolas, que posteriormente terão sua produção de grãos utilizadas no preparo de alimentos e rações destinadas a humanos e animais (Tassaneeyakul et al. 2004). Em regiões de clima tropicais e subtropicais, o desenvolvimento de fungos do gênero *Fusarium* é favorecido em condições de alta umidade e temperaturas entre 15 e 25°C (Mallmann & Dilkin 2007). Uma ampla gama de metabólitos secundários com estruturas químicas variadas pode ser produzidas por fungos toxígenos do gênero *Fusarium*, resultando na co-contaminação dos grãos por diversas micotoxinas (Grenier & Oswald 2011; Hussein & Brasel 2001).

Micotoxinas produzidas por fungos do gênero *Fusarium* incluem as fumonisinas, ácido fusárico, fusarinas, tricotecenos, zearelenona, moniliformina, entre outras. Fumonisina B_1 (FB_1), fumonisina B_2 (FB_2) e ácido fusárico (A.FUS) são as micotoxinas que apresentam maior importância toxicológica e ocorrência em grãos destinados à alimentação animal (Santin et al. 2000). No Brasil a ocorrência de fumonisinas em milho é constatada por diversos pesquisadores (Hirooka et al. 1996; Mallmann et al. 2001). No período de 2007 a 2011, o Laboratório de Análises Micotoxicológicas da Universidade Federal de Santa Maria analisou 19.914 amostras de milho, encontrando 82% de positividade com uma contaminação média geral de 2,19 mg/kg (LAMIC, 2012).

Devido a semelhança estrutural das fumonisinas com esfinganina e esfingosina, seu principal mecanismo de ação é a interferência no metabolismo dos esfingolipídios, sendo a relação esfinganina/esfingosina (SA/SO) utilizada como biomarcador da exposição as fumonisinas (Voss et al. 2007).

A presença de micotoxinas em grãos é associada a diversas síndromes em animais. Os equinos e suínos apresentam maior sensibilidade a fumonisina B_1, onde observam-se sinais clínicos de leucoencefalomalácia (LEME) e lesões clínicas de edema pulmonar, respectivamente (Mallmann et al. 1999; Dilkin 2002a). Frangos de corte parecem ser sensíveis a fumonisina B_1, ocasionando alterações no desempenho após consumo de ração contaminada. (Kubena et al. 1992; Ledoux et al. 1992).

O objetivo deste trabalho foi avaliar o desempenho de frangos de corte submetidos a dietas contendo materiais de cultivo fúngico produzidos a partir de quatro diferentes cepas toxígenas de fungos do gênero *Fusarium*, durante 21 dias.

MATERIAL E MÉTODOS

Solventes, reagentes e padrões analíticos

Todos os solventes e reagentes utilizados para realização das análises micotoxicológicas dos materiais de cultivo fúngico foram de graduação cromatográfica (Grau CLAE), marca J.T.BAKER obtidos de Sovereign, São Paulo, Brasil. Os padrões analíticos utilizados para quantificação das micotoxinas produzidas pelos cultivos fúngicos foram: fumonisina B_1, fumonisina B_2, fumonisina B_3 e ácido fusárico marca Sigma-Aldrich, obtidos de Servylab, Porto Alegre, Brasil. A água utilizada foi purificada através de osmose reversa com sistema Milli-Q (Millipore, EUA).

Materiais de cultivo fúngico

Os materiais de cultivo fúngico foram produzidos apartir de quatro diferentes cepas fúngicas toxígenas do gênero *Fusarium*, isoladas de casos clínicos suspeitos de fusariotoxicose, seguido da inoculação em câmaras de fermentação contendo milho, conforme procedimento descrito por Dilkin et al. 2002b.

Análises micotoxicológicas dos materiais de cultivo fúngico

Os materiais de cultivo fúngico foram analisados através de cromatografia líquida de alta eficiência acoplada a espectrometria de massas (CLAE-EM/EM), utilizando o método

descrito por Sulyok et al. (2006). Alíquotas de 1 g de material de cultivo fúngico foram pesadas em tubo de polipropileno e 20 mL de solução acetonitrila:água 84:16 (v/v) foram adicionados para extração das micotoxinas em mesa agitadora por 1 hora e 30 minutos. Após, as amostras foram filtradas e diluídas 1:1, (v/v) em solução acetonitrila:água:metanol 6:3:1, (v/v/v) contendo 1% de ácido acético e encaminhadas para a análise por CLAE-EM/EM.

Os fatores de recuperação das metodologias analíticas empregadas nas análises micotoxicológicas, foram obtidos através da realização de ensaios de recuperação, nos quais concentrações conhecidas de cada micotoxina foram adicionados a matriz, seguido a sua determinação através de CLAE-EM/EM (Ribani et al. 2004).

Experimentação animal

Seiscentos frangos de corte machos da linhagem Cobb 500 com um dia de vida, foram divididos em 5 tratamentos com 10 replicatas cada, com 12 aves em cada replicata. As aves foram mantidas em gaiolas com área de 0,25 m^2, dispostas em baterias, alocadas em salas com controle de temperatura e umidade através de condicionadores de ar e exaustores e iluminada do primeiro ao 21^o dia. As aves foram inspecionadas duas vezes ao dia para verificação da mortalidade.

Uma dieta basal formulada com milho e soja de acordo com os requerimentos nutricionais de frangos de corte (NRC, 1994) foi fornecida *ad libitum* a todos os grupos durante o período do experimento. Cada grupo (T1, T2, T3 e T4) recebeu a mesma dieta basal, com a incorporação do material de cultivo fúngico produzido por uma das quatro diferentes cepas fúngicas toxígenas, em quantidade necessária para atingir a concentração de 100 mg/kg de fumonisina B_1. As concentrações das demais micotoxinas foram calculadas a partir da quantidade de material de cultivo fúngico incorporado a cada dieta.

O protocolo realizado neste trabalho, incluindo a coleta de material biológico, foi revisado e aprovado pelo Comitê de Ética no Uso de Animais da Universidade Federal de Santa Maria sob o parecer número 025/2014.

Avaliação do desempenho

Todas as aves foram pesadas individualmente no início do experimento, sendo selecionadas aquelas que estivessem com peso entre 39 e 44 g, evitando dessa forma, diferença estatística significativa ($P < 0.05$) no peso inicial entre os tratamentos. Semanalmente, as aves foram pesadas individualmente para avaliação de desempenho. O

consumo de ração e a conversão alimentar (consumo de ração/ganho de peso) foram calculados nestes mesmos períodos.

Análises sorológicas

Aos 14 e 21 dias do experimento, 20 aves de cada tratamento (duas por replicata) foram eutanasiadas com utilização de dióxido de carbono para posterior coleta de sangue. As amostras de sangue foram centrifugadas por 30 minutos e o soro armazenado a -20^{o}C até a realização das análises. Os resultados das análises de esfinganina e esfingosina livre para avaliação da relação (SA/SO) foram realizadas através do procedimento descrito por Rauber et al. (2012). As análises de proteínas plasmáticas totais (PPT) foram realizadas utilizando *kits* comerciais obtidos de Labtest Diagnostica, SA, Vista Alegre, Minas Gerais, Brasil.

Análises estatísticas

Todos os dados obtidos neste experimento foram submetidos a análise de variância (one-way ANOVA) e as médias foram comparadas pelo teste de Bonferroni ($P \leq 0.1$), utilizando o *Software* Statgraphics Centurion (Statgraphics Centurion 15.2.14, Manugistics, Inc., Rockville, MD, EUA).

RESULTADOS E DISCUSSÃO

Análises micotoxicológicas

Os resultados das análises micotoxicológicas dos materiais de cultivo fúngicos são mostrados na tabela 1. Todas as cepas fúngicas toxígenas produziram fumonisinas B_1, B_2, B_3 e ácido fusárico.

Desempenho

A mortalidade dos frangos calculada ao final do experimento foi de 2% (12 aves). Os parâmetros de avaliação de desempenho são mostrados na tabela 2. O peso corporal médio das aves foi significativamente inferior ($P = 0.00$) aos 21 dias de experimento nos grupos que receberam dieta com incorporação de materiais de cultivo fúngico em relação ao grupo controle. O consumo de ração foi significativamente inferior ($P < 0.05$) nos grupos T1 e T3 em relação ao controle aos 21 dias. Conversão alimentar significativamente aumentada ($P = 0.00$) em relação ao controle foi observada em todos os grupos. Além disso, observou-se

diferença estatística ($P < 0.05$) na conversão alimentar do grupo T3 em relação ao grupo controle, T1 e T4.

Assim como neste trabalho, Weibking et al. (1993) observaram redução no peso corporal e consumo de ração em experimento com duração de 21 dias, no qual frangos de corte receberam dietas contendo 450 mg/kg de fumonisina B_1. Javed et al. (1993) observaram diminuição no ganho de peso e aumento na mortalidade em frangos de corte de diferentes idades submetidos a dieta contendo fumonisinas B_1 ou moniliformina. Além disso, efeitos aditivos foram observados quando estas micotoxinas foram administradas em combinação. Rauber et al. (2013) reportou diminuição no peso corporal e no consumo de ração em frangos de corte submetidos a dietas contendo 100 mg/kg de fumonisinas B_1 aos 14 dias de experimento, e somente aos 28 dias foi observada conversão alimentar aumentada.

A conversão alimentar calculada para o grupo T3, apresentou valor superior em decorrência do menor ganho de peso das aves que receberam dietas contendo materiais de cultivo fúngico. Esta diminuição no ganho de peso, possivelmente pode estar relacionada a interação entre o ácido fusárico e as fumonisinas, presentes no material de cultivo utilizado para preparo da dieta fornecida ao grupo T3. Após ingeridas, as fumonisinas podem ocasionar alterações morfológicas intestinais que influenciam a digestibilidade dos nutrientes presentes na ração, diminuindo seu aproveitamento com consequente prejuízo ao desempenho dos animais (Rauber et al. 2013; Voss et al. 2007). Ademais, o ácido fusárico parece influenciar o consumo de rações pelos animais devido a interferência na síntese de serotonina, neurotransmissor que atua na regulação do apetite (Magon et al. 2000). Bacon et al. (1995) reportou o efeito tóxico sinergístico em embriões de frango, quando o ácido fusárico em níveis não tóxicos foi administrado juntamente com fumonisina B_1, sugerindo que o ácido fusárico é responsável por aumentar a toxicidade de outras micotoxinas quando ingeridas de forma concomitante.

Análises sorológicas

Os resultados das análises de proteínas totais plasmáticas e a relação SA/SO são apresentados na tabela 3. O aumento dos níveis protéicos no soro das aves que receberam dietas com material de cultivo incorporado, foram diferentes significativamente ($P < 0.1$) somente no grupo T3 em relação ao grupo controle aos 21 dias. A relação SA/SO teve aumento significativo ($P = 0.00$) em todos os grupos em relação ao grupo controle aos 14 dias. Ademais, aos 21 dias de experimento houve aumento significativo ($P = 0.00$) na relação SA/SO do grupo T3 em relação ao grupo controle e T1.

CONCLUSÃO

Os resultados encontrados neste trabalho, demonstram que diferentes cepas toxígenas do gênero *Fusarium*, isoladas de casos clínicos suspeitos de fusariotoxicose, produzem concentrações variadas de micotoxinas sob as mesmas condições de cultivo. A possibilidade de co-ocorrência de micotoxinas requer que as possíveis interações entre elas sejam pesquisadas. Neste experimento, os resultados observados indicam que 100 mg/kg de fumonisina B_1 em presença de ácido fusárico em níveis acima de 80 mg/kg afetam significativamente o desempenho de frangos de corte aos 21 dias.

Agradecimentos – Ao Frigorífico AVISUI Alimentos a disponibilização das instalações para realização das etapas de coleta de material e eutanásia das aves; a Coordenação de Aperfeiçoamento de Pessoal de Nível de Superior (CAPES), a concessão de bolsa de estudos ao primeiro autor.

BIBLIOGRAFIA

Bacon C.W., Porter J.K. & Norred W.P. 1995. Toxic interaction of fumonisin B_1 and fusaric acid measured by injection into fertile chicken egg. Mycopathologia 129:29-35.

Mallmann C.A., Santurio J.M. & Dilkin P. 1999. Equine leukoencephalomalacia associated with ingestion of corn contaminated with fumonisin B_1. R. Microbiol. 30:249-252.

Dilkin P. Micotoxicose suína: aspectos preventivos, clínicos e patológicos. 2002a. Biológico, São Paulo. 64(2):187-191.

Dilkin P., Mallmann C.A., Almeida C.A.A., Stefanon E.B., Fontana F.Z. & Milbradt E.L. 2002b. Production of fumonisins by strains of *Fusarium verticillioides* according to temperature, moisture and growth period. Braz. J. Microbiol. 33:111-118.

Dall'Asta, C., Galaverna, G., Aureli, G., Dossena, A. & Marchelli, R. 2008. A LC/MS/MS method for the simultaneous quantification of free and masked fumonisins in maize and maize-based products. World Mycotoxin Journal 1:237-246.

EFSA (European Food Safety Authority) 2007. Opinion of the Scientific Panel on contaminants in the food chain related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. EFSA J. 446:1–127.

Grenier, B. & Oswald, I.P. 2011. Mycotoxin co-contamination of food and feed: meta-analysis of publications describing toxicological interactions. World Mycotoxin Journal. 4:285-313.

Hirooka E.Y., Yamaguchi M.M., Aoyama S., Sugiura Y. & Ueno Y. 1996. The natural occurrence of fumonisins in Brazilian corn kernels. Food Addit. Contam. 13:173-183.

Hussein H.S., & Brasel J.M. 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology, 167:101-134.

Javed T., Bennett G.A., Richard J.L., Dombrink-Kurtzman M.A., Cote L.M. & Buck W.B. 1993. Mortality in broiler chicks on feed amended with *Fusarium proliferatum* culture material or with purified fumonisin B_1 and moniliformin. Mycopathologia 123:171–184.

Marin S., Ramos A.J., Cano-Sancho G., Sanchis V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. Food and Chemical Toxicology. 60:218-237.

Kubena L.F., Edrington T.S., Harvey R.B., Buckley S.A., Phillips T.D., Rottinghaus G.E. & Casper H.H. 1992. Individual and Combined Effects of Fumonisin B_1 Present in

Fusarium moniliforme Culture Material and T-2 Toxin or Deoxynivalenol in Broiler Chicks. Poultry Science 76:1239–1247.

LAMIC. Laboratório de Análises Micotoxicológicas, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil. Tabelas de resultados, 2012. Disponível em: <http://www.lamic.ufsm.br>. Acesso em: 29 jan. 2012.

Ledoux D.R., Brown T.P., Weibking T.S., & Rottinghaus G.E. 1992. Fumonisin toxicity in broiler chicks. Journal of Veterinary Diagnostic Investigation. 4:330-333.

Mallmann C.A. & Dilkin P. 2007. Micotoxinas e Micotoxicoses em Suínos. Editora Soc. Vic. Pallotti, Santa Maria, 240 p.

Mallmann C.A.; Santurio J.M., Almeida C.A.A. & Dilkin P. 2001. Fumonisin B1 levels in cereals and feeds from Southern Brazil. Arq. Inst. Biol., v. 68:41-45.

NRC 1994. Nutrient Requirement of Poultry. 9th ed. Natl Acad. Press, Washington, DC.

Rauber R.H., Dilkin P., Mallmann A.O., Marchioro A., Mallmann C.A., Borsoi A. & Nascimento V.P. 2012. Individual and combined effects of *Salmonella typhimurium* lipopolysaccharide and fumonisin B_1 in broiler chickens. Poultry Science. 91:2785-2791.

Ribani M., Bottoli C.B.G., Collins C.H., Jardins I.C.S.F. & Melo L.F.C. 2004. Validação em métodos cromatográficos e eletroforéticos. Quim. Nova. 27(5):771-780.

Santin E., Maiorka A., Zanella I. & Magon L. 2000. Micotoxinas do *Fusarium spp* na avicultura comercial. Ciência Rural. 31(1),185-190.

Sulyok, M., Berthiller, F., Krska, R., & Schuhmacher, R. 2006. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. Rapid Communications in Mass Spectrometry, 20(18), 2649-2659.

Tassaneeyakul W., Razzazi-Fazeli E., Porasuphatana S., Bohm J., 2004. Contamination of aflatoxins in herbal medicinal products in Thailand. Mycopathologia. 158:239–244.

Voss K.A., Smith G.W. & Hascheck W.M. 2007. Fumonisins: Toxicokinectics, mechanism of action and toxicity. Animal Feed Science and Technology. 137:299-325.

Tabela 1 – Análises micotoxicológicas dos materiais de cultivo fúngico de *Fusarium*

Cultivo	Concentração (mg/kg)			
	FB_1 [a]	FB_2 [b]	FB_3 [c]	A.FUS [d]
C1	6720,0	1344,2	940,8	2354,1
C2	6660,0	1132,1	965,7	986,5
C3	5590,0	894,4	838,5	5239,7
C4	4930,0	936,7	640,9	3912,1
Recuperação	98,1	96,2	95,2	97,1

[a] FB_1= fumonisina B_1; [b] FB_2= fumonisina B_2; [c] FB_3= fumonisina B_3; [d] A.FUS= ácido fusárico.

Tabela 2 – Parâmetros de desempenho avaliados em frangos de corte submetidos a dietas contendo diferentes materiais de cultivo fúngico

Grupo	Peso corporal (CV[a])		Consumo de ração (CV)		CA[b] (CV)	
	14 dias	21 dias	14 dias	21 dias	14 dias	21 dias
Controle	425.2^{A} (13.2)	822.6^{A} (12.1)	518.1^{A} (4.2)	1159.5^{A} (4.1)	1.20^{B} (4.1)	1.41^{C} (2.3)
T1	400.3^{B} (13.1)	732.5^{B} (13.9)	493.5^{A} (4.1)	1100.4^{B} (4.2)	1.23^{AB} (3.7)	1.50^{B} (4.4)
T2	398.7^{B} (17.3)	734.6^{B} (17.4)	498.1^{A} (4.8)	1103.2^{B} (3.2)	1.26^{AB} (5.4)	1.52^{AB} (6.2)
T3	400.9^{B} (10.8)	718.5^{B} (16.0)	512.7^{A} (6.6)	1152.7^{AB} (3.8)	1.28^{A} (4.6)	1.61^{A} (6.1)
T4	409.9^{AB} (10.8)	754.6^{B} (12.8)	503.9^{A} (5.1)	1130.4^{AB} (4.2)	1.23^{AB} (5.3)	1.50^{B} (6.4)
P_{anova}	<0.00	0.00	0.19	0.01	0.04	0.00

[A-C] Médias com letras maiúsculas na mesma coluna diferem pelo teste de Bonferroni (P≤0.1) [a] CV= Coeficiente de variação (%). [b] CA = Conversão alimentar (consumo de ração/ganho de peso).

Tabela 3 – Análises sorológicas realizadas em frangos de corte submetidos a dietas contendo material de cultivo fúngico

Grupo	PPT[a] (CV[b])		SA:SO[c] (CV)	
	14 dias	21 dias	14 dias	21 dias
Controle	2.86^{A} (14.1)	2.98^{B} (12.1)	0.74^{B} (21.1)	0.88^{C} (19.1)
T1	3.07^{A} (12.4)	3.21^{AB} (8.8)	1.45^{A} (15.1)	1.51^{B} (14.1)
T2	3.13^{A} (10.0)	3.26^{AB} (9.3)	1.51^{A} (18.2)	1.57^{AB} (22.4)
T3	3.24^{A} (11.3)	3.32^{A} (10.5)	1.63^{A} (16.2)	1.85^{A} (17.2)
T4	2.98^{A} (9.7)	3.21^{AB} (7.1)	1.58^{A} (21.4)	1.64^{AB} (20.9)
P_{anova}	NS[d]	0.05	0.00	0.00

[A-C] Médias com letras maiúsculas na mesma coluna diferem pelo teste de Bonferroni (P≤0.1)

[a] PPT= Proteínas plasmáticas totais; média dos níveis no soro (g/dL). [b] CV= Coeficiente de variação (%). [c] SA/SO= relação média dos níveis de esfinganina/esfingosina no soro. [d] Resultado não mostrou diferença significativa pelo teste de Bonferroni (P≤0.1).

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Identificação de micotoxinas em cultivos fúngicos do gênero *Fusarium* e sua influência no desempenho produtivo de frangos de corte"

Número do Parecer: 025/2014

Pesquisador Responsável: Prof. Dr. Carlos Augusto Mallmann

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS:
É necessário que as informações e alterações realizadas na apresentação de pendências sejam acrescidas ao formulário unificado que deve ser ressubmetido à CEUA;
A CEUA se baseia nas recomendações do CONCEA em suas Diretrizes Da Prática de Eutanásia, publicadas em de 2013 (http://www.mct.gov.br/upd_blob/0226/226746.pdf).
Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.
Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DE APROVAÇÃO: 02/06/2014

Santa Maria, 02 de junho de 2014.

Prof. Dr. Alexandre Krause
Coordenador da Comissão de Ética no Uso de Animais - UFSM

CONCLUSÕES

ARTIGO 1

- A co-ocorrência de diversas micotoxinas em amostras de milho coletadas nos estados da região Sul do Brasil é constatada.
- Todas as amostras de milho analisadas estão contaminadas com fumonisina B_1 e fumonisina B_2.
- Entre as amostras analisadas, todas as micotoxinas que possuem limites máximos permitidos estabelecidos pela legislação brasileira estão presentes, com exceção da ocratoxina A.
- Algumas amostras de milho apresentam concentrações de micotoxinas acima dos limites máximos estabelecidos pela legislação.
- Metabólitos secundários, não regulamentados pela legislação brasileira, produzidos por fungos do gênero *Aspergillus*, *Fusarium*, *Alternaria*, *Penicillium*, além de metabólitos produzidos por bactérias, são detectados em amostras de milho.

ARTIGO 2

- Altas concentrações de fumonisinas ligadas ocorrem em amostras de milho
- Fumonisinas ligadas são detectadas em amostras de milho onde fumonisinas livres não são detectadas.
- As concentrações de fumonisinas livres e ligadas apresentam correlação positiva.

ARTIGO 3

- A presença de fumonisinas e ácido fusárico na dieta de frangos de corte, a partir do primeiro dia de vida, até o 21° dia, determina efeitos negativos significativos sobre o desempenho das aves.
- Consumo de ração é significativamente inferior nos grupos que recebem dietas contendo material de cultivo fúngico, em relação ao controle.

- Conversão alimentar é significativamente superior em todos os grupos em relação ao controle. Além disso, observa-se diferença estatística na conversão alimentar entre grupos submetidos a dieta incorporada de material de cultivo fúngico.
- Os níveis de proteínas plasmáticas totais é superior nos grupos submetidos a dieta contaminada com material de cultivo fúngico em relação ao grupo controle.
- A relação entre esfinganina e esfingosina séricas (SA/SO) é alterada pela intoxicação com fumonisinas e ácido fusárico.

REFERÊNCIAS

ABIA, W. A., WARTH, B., SULYOK, M., KRSKA, R., TCHANA, A. N., NJOBEH, P. B., DUTTON, M. F., & MOUNDIPA, P. F. Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). **Food Control**, v.31, n.2, p.438-453, 2013.

BHAT, R., RAI, R. V. & KARIM A. A. Mycotoxins in Food and Feed: Present Status and Future Concerns. **Comprehensive Reviews in Food Science and Food Safety.** v.9, p.57-81, 2010.

BENNETT, J. W. & KLICH, M. Mycotoxins. **Clinical Microbiology Reviews.** v.16, p.497–516, 2003.

BERTHILLER, F., SCHUHMACHER, R., ADAM, G., & KRSKA, R. Formation, determination and significance of masked and other conjugated mycotoxins. ***Analytical and Bioanalytical Chemistry***, v.395, n.5, p.1243-1252, 2009.

BERTHILLER, F., CREWS, C., DALL'ASTA, C., SAEGER, S. D., HAESAERT, G., KARLOVSKY, P., et al. Masked mycotoxins: a review. **Molecular Nutrition & Food Research**, v.57, n.1, p.165-186, 2013.

BRASIL, 2011. Agência Nacional de Vigilância Sanitária (ANVISA). Resolução RDC No 7, de 18 de fevereiro de 2011. Dispõe sobre limites máximos tolerados (LMT) para micotoxinas em alimentos. **Diário Oficial da União** – Ministério da Saúde, Brasília, DF, Brasil, de 22/03/2011. Dísponivel em: <http://portal.anvisa.gov.br/wps/wcm/connect/bc17db804f45fe2cbd41fdd785749fbd/Resolu%C3%A7%C3%A3o+0-2011-CGALI.pdf?MOD=AJPERES>. Acesso em: 10 jun. 2014.

FRISVAD, J. C., THRANE, U. & SAMSON, R. A. Mycotoxins producers. In:____. **Food m ycology: A Multifaceted Approach to Fungi and Food.** Boca Raton: CRC Press. 2007. Part 3, p. 135–163.

GOMPERTZ, O. F.; GAMBALE, W.; PAULA, C. R. **Características gerais dos fungos.** In: TRABULSI, L. R.; ALTERTHUM, F. Microbiologia. 5 ed. São Paulo: Atheneu. 2008. p.479-491.

GRENIER, B. & OSWALD, I. P. Mycotoxin co-contamination of food and feed: meta-analysis of publications describing toxicological interactions. **World Mycotoxin Journal**, v.4, 285-313, 2011.

HUSSEIN, H. S. & BRASEL, J. M. Toxicity, metabolism, and impact of mycotoxins on human and animals. **Toxicology**. v.67. p.101-134, 2001.

IARC. 1993. Toxins derived from *Fusarium graminearum*, *F. culmorun* and *F. crookwellense*: zearalenone, deoxynivalenol, nivalenol and fusarenone x (Group 3). **IARC Monographs on the Evaluation of Carcinogenic Risks to Humans**, v.56, p.397.

IARC. 2002. Traditional herbal medicines, some mycotoxins, naphtalene, and styrene. **IARC Monographs on the Evaluation of Carcinogenic Risks to Humans**, v.82, p.169-366.

MAGAN, N. & ALDRED, D. Why do Fungi produce mycotoxins. In:____. **Food m ycology: A Multifaceted Approach to Fungi and Food.** Boca Raton: CRC Press. 2007. Part 3, p.212–135.

MALLMANN, C. A.; DILKIN, P. **Micotoxinas e Micotoxicoses em Suínos.** 1 ed. Santa Maria: Ed. Do Autor, 2007. 240 p.

SMITH, T. K. & SEDDON, I. R. 1998. **Toxicological synergism between *Fusarium* mycotoxins in feeds**. In: Lyons TP, Jacques KA, editors. Biotechnology in the feed industry. Loughborough, U.K.:Nottingham Univ. Press. 1998. p.257–269.

SULYOK, M., BERTHILLER, F., KRSKA, R., & SCHUHMACHER, R. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. **Rapid Communications in Mass Spectrometry**, v.20, n.18, p.2649-2659, 2006.