Assessing Immunomodulatory Effects of *Penicillium* Mycotoxins using Bovine Macrophages Cell Line

by

Se-Young Oh

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ABSTRACT

Assessing Immunomodulatory Effects of *Penicillium* Mycotoxins using Bovine Macrophages Cell Line

Se-Young Oh  
University of Guelph, 2014

Advisor:  
Dr. Niel A. Karrow

Co-Advisor:  
Dr. Herman J. Boermans

The present study assessed the immunomodulatory effects on macrophages of the following *Penicillium* mycotoxins (PMs), including citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophilic acid (MPA) and penicillic acid (PA), by using a bovine macrophage cell line (BoMacs). Initially, concentration-response curves for each of these PMs were established based on cell proliferation and viability. The potency of these PMs based on their IC50s (concentration that inhibits 50% cell proliferation) from highest to lowest was: 0.56 µM (PAT) > 12.88 µM (OTA) > 29.85 µM (PA) > 91.20 µM (CIT) > not determined (MPA). LC50s (concentrations that kill 50% of cells) for PAT and PA were determined to be 4.46 µM and 175.79 µM, respectively. In addition to this, binary mixtures of some PMs at their respective IC25 and lower were shown to have significant interactions on cell proliferation. CIT+OTA had significant synergism, while PAT+MPA, CIT+MPA, CIT+PA and MPA+PA exhibited significant antagonism. PAT+PA showed significant antagonism at their respective IC25s, but were antagonistic at lower concentrations. The PMs at their sub-lethal levels of IC25s also differentially altered the gene expression of the following cytokines, including *IL-1α, IL-6, IL-10, IL-12, IL-23* and *TGF-β*. Reactive oxygen species (ROS) production and phagocytosis of *Mycobacterium avium* subspecies...
paratuberculosis (MAP) was affected at higher sub-lethal concentrations. OTA in combination with CIT or PA synergistically suppressed the gene expression of the following epigenetic enzymes: DNA methyltransferases (DNMT-3s), histone demethylase (JMJD-3) and histone deacetylase (HDAC-3). When the efficacy of a mycotoxin-binding Mycosorb A+ (MA+), a mycotoxin binder, was assessed based on BoMac proliferation, 0.2% MA+ showed the highest efficacy in preventing OTA toxicity at pH 3.0, while its beneficial effect in preventing CIT toxicity was also observed with a longer incubation time than 6 hours and higher inclusion level of MA+ than 0.5%. The results from this thesis indicate that the PMs at their sub-lethal concentrations could potentially modulate the macrophage functions. These adverse effects also appear to be enhanced by PM interactions. This study also showed beneficial effect of MA+ in preventing the toxicity of PMs.
ACKNOWLEDGEMENTS

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>0.25IC25</td>
<td>Second serial dilution of IC25</td>
</tr>
<tr>
<td>0.5IC25</td>
<td>First serial dilution of IC25</td>
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<tr>
<td>A. Aspergillus</td>
<td>Antibody-mediated immune response</td>
</tr>
<tr>
<td>AbMIR</td>
<td>Antibody-mediated immune response</td>
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<tr>
<td>ADH</td>
<td>Yeast alcohol dehydrogenase</td>
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<td>BALF</td>
<td>Bronchioalveolar lavage fluid</td>
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<td>B-cell</td>
<td>B-lymphocytes</td>
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<td>Bmi-1</td>
<td>Polycomb ring finger oncogene</td>
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<td>BoMacs</td>
<td>Bovine macrophage cell line</td>
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<td>CA</td>
<td>Concentration addition</td>
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<td>Caco-2</td>
<td>Human epithelial colorectal adenocarcinoma cells</td>
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<td>CCR</td>
<td>Chemokine receptors</td>
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<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>CIT</td>
<td>Citrinin</td>
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<td>CMIR</td>
<td>Cell-mediated immune response</td>
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<td>CO2</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
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<td>FAO</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>Foxp3</td>
<td>Forkhead Box P3</td>
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<td>GALT</td>
<td>Gut-associated lymphoid tissues</td>
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<td>GAPDH</td>
<td>Glyceraldheyde 3-phosphate dehydrogenase</td>
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<td>GDP</td>
<td>Guanosine Diphosphate</td>
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<td>GMA</td>
<td>Glucamannan mycotoxin adsorbent</td>
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<td>H2DFFDA</td>
<td>5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate</td>
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<td>Hydrogen Peroxide</td>
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<td>H3K27</td>
<td>Histone H3 lysine 27</td>
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<td>Histone deacetylases</td>
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<td>HDACi</td>
<td>Histone deacetylase inhibitors</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
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<td>HPLC</td>
<td>High performance Lipid Chromatography</td>
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<td>hsp72</td>
<td>Heat shock protein 72</td>
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<td>IκB-α</td>
<td>Inhibitor of NF-κB</td>
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<td>IA</td>
<td>Independent action</td>
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<td>Concentration that inhibits 25% of cell proliferation</td>
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<tr>
<td>LC</td>
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<td>mDC</td>
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<td>MOI</td>
<td>Multiplicity of infection</td>
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<td>NO</td>
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<td>OD</td>
<td>Optical Density</td>
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<td>Description</td>
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<td>Percent proliferation from the individual mycotoxins</td>
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<td>Pathogen-associated molecular patterns</td>
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<td>Patulin</td>
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<td>Peripheral blood mononuclear cell</td>
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<td>P_OM</td>
<td>Observed percent proliferation from the mixture</td>
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<td>PRRs</td>
<td>Pattern recognition receptors</td>
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<td>PWM</td>
<td>Pokeweed mitogen</td>
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<td>qPCR</td>
<td>Quantitative PCR</td>
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<td>RBCs</td>
<td>Red blood cells</td>
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<td>RNA</td>
<td>Ribodeoxynucleic acid</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>RPMI 1640</td>
<td>Roswell Park Memorial Institute 1640 media</td>
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<td>RQC</td>
<td>Roquefortine C</td>
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<td>Spp</td>
<td>Species</td>
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<tr>
<td>sRBCs</td>
<td>Sheep red blood cells</td>
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<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
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<tr>
<td>T-2 toxin</td>
<td>Trichothecene mycotoxin</td>
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<td>T-cell</td>
<td>T-lymphocytes</td>
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<td>TGF-β</td>
<td>Transforming growth factor</td>
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<td>TLC</td>
<td>Thin Layer Chromatography</td>
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<td>TNF-α receptors-1</td>
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<td>Tumor necrosis factor-α</td>
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<td>TNF receptor-1-associated death domain</td>
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<td>Regulatory T-cells</td>
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<tr>
<td>UTX</td>
<td>Tetratricopeptide-repeat X</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
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Chapter 1: Literature Review

1.1. Introduction

1.1.1. General Overview

Mycotoxins are biologically active secondary metabolites of filamentous fungi that commonly contaminate a wide variety of cereal and livestock feed crops worldwide. The Food and Agriculture Organization (FAO) estimates that approximately 25% of food and foodstuffs around the world are contaminated by fungal mycotoxins (Boutrif and Canet, 1998). Increasing humidity and temperature accompanying global climate change will likely favour more fungal contamination of feedstuffs, affecting the safety of both human and animal feeds (Miraglia et al., 2009). Economic losses from mycotoxin contamination occur due to reduced crop yield and value, as well as decreased livestock productivity and health from the consumption of contaminated feed (Schmalle III and Munkvold, 2009).

Penicillium molds are one of the common fungal species found in crops, fruits and animal feeds of Northern Europe, North and South America as well as East Asia (Almeida et al., 2000; Creppy, 2002; Duong, 1996; Xiao et al., 2011). Poor storage conditions for most grains and food products often provide favourable environmental conditions, such as moderate temperature with high humidity, for mycotoxin production from Penicillium mold species (Dalcero et al., 1998; Mansfield et al., 2008). Due to their dramatic increase during the ensilaging of animal feed, the Penicillium mycotoxins (PMs) are also referred as ‘silage mycotoxins.’

A number of PMs can be detected in stored feed or food such as ochratoxin (OTA), citrinin (CIT), patulin (PAT), penicillic acid (PA), mycophilic acid (MPA), and roquefortine C (RQC) (Lennox et al., 2003; Mateo et al., 2007). These PMs are known to cause a variety of toxic effects in animals. For example, OTA, CIT and PA are nephrotoxic (Braunberg et al., 1992; Sansing et
and hepatotoxic (Dickens and Jones, 1965) in monogastric species, while PAT is a potent hepatotoxicant in both monogastric and ruminant species (Hayes et al., 1979; Sommer et al., 1974; Ukai et al., 1954). These PMs also affect the host immune response, which could predispose animals to secondary diseases (Table 1.1 and 1.2; Al-Anati and Petzinger, 2006; Ferrante et al., 2008; Herzog-Soares and Freire, 2004).

Regardless of their abundance in animal feed and their potential to affect the immune system, the agricultural and economic importance of PMs are often under-estimated because it is generally assumed that PMs are readily metabolized by gut microbiota and hepatic enzymes (Abrunhosa et al., 2010; Fuchs et al., 2008). However, considering that most of these PMs have anti-microbial properties (Kavanagh, 1947) and are found to be hepatotoxic to animals (Dickens and Jones, 1965; Hayes et al., 1979; Sansing et al., 1976), it is possible that detoxification of the PMs is attenuated by these properties. RQC for example, was found to inhibit cytochrome-p450 activity by reducing nicotinamide adenine dinucleotide phosphate (NADPH) consumption (Aninat et al., 2001), and p450 is an important enzyme for detoxifying mycotoxins (Smith and Harran, 1993). Furthermore, mycotoxins can also enter the circulation and target tissues without passing though the digestive tract. For example, it has been reported that some *Fusarium* and/or *Penicillium* mycotoxins, such as OTA, can enter the circulation during inhalation (Halstensen et al., 2004; Skaug et al., 2001) and dermal exposure (Panda et al., 1997). Since entry via these routes bypasses initial detoxification provided by gut microbiota and the liver, exposure via inhalation and dermal contact could be a health risk for farmers and livestock (EFSA, 2012; Reijula and Tuomi, 2003; Smith et al., 1995).
1.1.2. The general occurrence and toxicity of PMs

1.1.2.1 Ochratoxin A (OTA)

OTA is a common PM found in stored cereal grains, nuts as well as some brewed liquors, because temperature and humidity during storage and brewing often favour OTA production (Mateo et al., 2007; Visconti et al., 1999). OTA was initially isolated from Aspergillus ochraceus in 1965, but was later also isolated from P. citrinin and P. verrucosum (Pitt, 1987). The lethal dose that kills 50% (LD50) of mice via intraperitoneal and oral exposure was estimated to be 29.4 mg/kg and 46.0 mg/kg, respectively (Moroi et al., 1985), which are much higher than typical OTA concentrations found in silage.

Nephrotoxicity is the most common pathology associated with OTA exposure at a concentration greater than 0.05 mg/kg in monogastric species, such as rats and pigs (Baudrimont et al., 1994; Milićević et al., 2008). However, a high dose of 13 mg/kg, or cumulative exposure to a concentration as low as 3 mg/kg was also reported to cause teratogenicity to embryos of rats and chicks (Brown et al., 1976; O’Brien et al., 2005; Stoev, 2010) as well as goats (Ribelin et al., 1978). A few studies have also shown that OTA significantly reduced the overall performance of both laying hens and broiler chickens (Niemiec and Borzemska, 1994; Prior et al., 1980). OTA was also found to have synergistic toxicity in poultry species when mixed with either CIT or PA (Stoev et al., 2004; Veselá et al., 1983).

1.1.2.2. Citrinin (CIT)

CIT is a common mycotoxin isolated along with the natural yellow biopigment produced by Monascus spp. (Carvalho et al., 2005). CIT was first isolated from P. citrinin (Hetherington and Raistrick, 1931), and was subsequently detected from P. verrucosum and P. expansum that
frequently contaminate cereal grains, rotten fruits, and juice (Martins et al., 2002; Scott et al., 1972).

Several animal studies have reported significant toxicity mediated by CIT, and the oral LD50 to the rat was estimated to be 50 mg/kg (Leatherhead RI, 2013). The LD50s in response to intraperitoneal exposure have been estimated to be 35-58 mg/kg for the mouse and 19 mg/kg for the rabbit (Leatherhead RI, 2013). CIT appears to be a nephrotoxin based on its toxicity to porcine kidney cells (Braunberg et al., 1992), while teratogenicity was also observed in rats that were exposed to 35 mg/kg during gestation and in chicken embryos exposed to CIT concentrations between 1.0 to 10.0 μg (Reddy et al., 1982; Veselá et al., 1983). The toxicity of CIT was shown to be synergistically enhanced in poultry species when co-exposure occurred with OTA (Veselá et al., 1983).

1.1.2.3. Patulin (PAT)

PAT is one of the most potent mycotoxins found in food (Puel et al., 2010). *P. expansum* is the most common mold species that produces PAT (Sommer et al., 1974); however, other *Penicillium* and *Aspergillus* mold species including *P. urchiae*, *P. melinii*, *P. claviforme*, *A. clavatus* and *A. terreus* can also produce PAT (Ciegler, 1977). PAT can be found in fruits, flour, cereal grains, and meat (Ciegler, 1977; Martins et al., 2002); however, it is most often detected in the fruit-based products, such as apple and grape juices (Kataoka et al., 2009; Scott and Somers, 1968).

The LD50 of acute PAT exposure for mice, neonatal rats, and weaning rats was determined to be 17, 6.7 and 108-118 mg/kg BW, respectively, with the following pathological signs: focal hepatic necrosis, atelectasis, alveolar septal congestion and intra-alveolar haemorrhaging (Hayes et al., 1979). In 1954, it was suggested that PAT caused haemorrhagic syndrome and the death of
over 100 Japanese dairy cattle that had been fed malt contaminated with PAT produced from *P. urticae* (Ukai et al., 1954). A similar incident occurred in Germany and France as a result of PAT produced from *A. clavatus* contamination of wheat (Ciegler, 1977). Chronic exposure to 0.1 mg/kg of PAT in rat feed resulted in various pathological signs, including haemorrhage, plasma cell hyperplasia as well as cell destruction and abnormalities of the nucleus and organelles of rat liver and kidneys (Arzu Koçkaya et al., 2009). No evidence of either teratogenicity or mutagenicity has been observed in mice (Reddy et al., 1978).

1.1.2.4. Penicillic acid (PA)

PA is often detected in various grains, tobacco, and has even been found in moldy sausages (Ciegler, 1977; Ciegler and Kurtzman, 1970; Müller and Amend, 1997; Snow et al., 1972). Its potential toxicity has been known since 1896; however, it was first isolated from *P. puberulum* in 1913 (Ciegler et al., 1971). Several different *Penicillium* spp. including *P. puberulum*, *P. martensii*, *P. palitans*, and *P. cyclopium* are the major producers of PA (Alsberg and Black, 1913; Ciegler and Kurtzman, 1970), but it is also produced by a few *Aspergillus* spp. such as *A. ochraceus* (Ciegler et al., 1972).

The LD50s for PA for rats via subcutaneous, intravenous and oral exposure were determined to be 110, 250 and 600 mg/kg, respectively (Murnaghan, 1946). PA has been shown to be hepatotoxic and hemorrhagic in dogs (Hayes et al., 1977), and potential carcinogenicity was reported in early rat and mice PA studies (Dickens and Jones, 1965). Similar to CIT, PA has been reported to be nephrotoxic and hepatotoxic to mice, and its toxic effects can be synergistically enhanced when mixed with OTA (Sansing et al., 1976).
1.1.2.5. Mycophenolic acid (MPA)

MPA is a common contaminant found in ensilaged and grain feeds (Burkin and Kononenko, 2010), as well as some of fermented dairy products (Usleber et al., 2008). MPA is produced by *P. stoloniferum*, *P. brevicompactum*, *P. roqueforti* and *P. carneurn* (Bartman et al., 1981; Boysen et al., 1996; Lafont et al., 1979).

LD50s for MPA by oral exposure were determined to be 352 mg/kg for the rat, 1000 mg/kg for the mouse, and over 6000 mg/kg for the rabbit (Menon et al., 2011), which indicates that MPA has relatively low toxicity in animals compared to other PMs. Although low in toxicity, one of the most distinctive characteristics of MPA is its anti-proliferative effect on specific cell types. MPA has been shown to inhibit the proliferation of smooth muscle cells (Ahn et al., 2007), endothelial cells (Mohacsi et al., 1997), as well as some immune cells, such as lymphocytes (Keblys et al., 2004; Oh et al., 2012). Ironically, MPA’s low toxicity and immunomodulatory properties have resulted in many human pharmaceutical studies, but few animal studies have been carried out despite the fact that it is frequently found at high concentrations in ensiled animal feed (Mansfield et al., 2008). MPA has been used therapeutically for the treatment of arteriosclerosis and rheumatoid arthritis (Cohn et al., 1999; Mohacsi et al., 1997), and to prevent organ rejection following kidney and heart transplantation (Weber et al., 1998).

1.1.2.6. Roquefortine C (RQC)

RQC is a major mycotoxin produced from *P. Roqueforti*, which is used to manufacture ‘Roquefort’ or ‘blue’ cheese. Other *Penicillium* spp. found in blue cheese or milk, including *P. commune*, *P. crustosum*, *P. chrysogenum*, *P. cyclopium* and *P. glabrum* are also capable of producing RQC (Häggblom, 1990; Rand et al., 2005; Wagener et al., 1980). The presence of RQC is very common in blue cheese products, yet it is considered to be safe for human consumption
due to the low exposure range (0.05 - 10 mg/kg food) and its low toxicity (Finoli et al., 2001). In contrast, however, RQC is found at relatively higher concentrations (up to 17 mg/kg) in moldy animal feed grains and silage, especially in the maize-based silage (Auerbach et al., 1998; Häggblom, 1990a; Ohmomo et al., 1994).

Despite its relatively low toxicity, it has been reported that natural RQC exposure concentrations may be high enough to cause potential toxicity to both monogastrics (Puls and Ladyman, 1988) and ruminants (Häggblom et al., 1990b). The LD50 for RQC in the mouse by intraperitoneal exposure was previously estimated to be 20 mg/kg (Ohmomo et al., 1975), but Tiwary et al. (2009) later re-determined the LD50 via intraperitoneal exposure to be between 169-184 mg/kg for mice. The pathology of RQC toxicity includes paralysis, trembling and lethargy in canine and murine species (Rand et al., 2005; Scott and Kennedy, 1976; Wagener et al., 1980). There are no reports of lethality in ruminant species, but there is a study reporting various clinical signs, including paresis, indigestion, diarrhea as well as increased rate of mastitis and abortion from the dairy cows fed 25 mg/kg of RQC (Häggblom et al., 1990b).

1.1.3. Effect of PMs on physical barriers

Epithelial cells and commensal microbes create an important physical barrier at mucosal surfaces that protect the host against pathogenic microbes and toxins. Epithelial cells form a mechanical and bioactive barrier providing attachment surfaces for commensal microbes, while the commensal microbes maintain a symbiotic relationship with the host to construct a protective barrier between the outside gut environment and host immune system (Yan and Polk, 2004; Yu et al., 2012). PMs could potentially disrupt these physical barriers by altering commensal microbial populations and destabilizing the epithelial cell barrier.
1.1.3.1. Disruption of commensal microbial populations

Hundreds of different microbial species reside within the GIT of monogastrics and ruminants where they maintain a symbiotic relationship with the host. These commensal microbes form a barrier on epithelial surfaces preventing attachment and colonization of pathogenic microbes (Croswell et al., 2009). Likewise, the biodiversity of these commensal populations is crucial for maintaining gut homeostasis, integrity and normal function of immune system (Magalhaes et al., 2007; Tappenden and Deutsch, 2007) (Figure 1.1A). For example, colonization of the gut lumen with *Escherichia coli*, *Klebsiella pnemoniae*, and *Streptococcus viridans* increases paracellular permeability, whereas, *Lactobacillus brevis* has the opposite effect (García-LaFuente et al., 2001).

It is hypothesized that when commensal microbial populations are altered, protective and homeostatic interaction between host and commensals are impaired, and this can lead to disease, such as inflammatory bowel disease (Schütze et al., 2010; Wagner et al., 2002).

The biodiversity of commensal microbiota is especially important for the development and maturation of neonatal immune system. Rapid colonization of the neonatal gut by commensal bacteria for example, leads to hypertrophy of Peyer’s patches, and attenuates the responsiveness of B cells to produce secretory IgA against commensal microbes (Shroff et al., 1995). Furthermore, specific commensal bacterial species promote the development of mucous-associated lymphoid tissues (Rhee et al., 2004), as well as immune system maturation to facilitate immunological tolerance to mucosal commensals and effector function against pathogenic microbes (Tourneur and Chassin, 2013). Interestingly, both delayed and inappropriate colonization of mucosal surfaces appears to be a risk factor for neonatal disease (Tourneur and Chassin, 2013).

Several PMs, such as CIT, PAT, MPA, PA and RQC, possess antimicrobial properties (Aninat et al., 2001; Kavanagh, 1947), which could disrupt commensal biodiversity and population numbers (Figure 1.1B). For example, CIT has an antimicrobial effect against several pathogenic
strains of bacteria and fungi (Devi et al., 2009), while PA has antimicrobial activities against Gram-positive bacterial species (Kavanagh, 1947). The antimicrobial properties of PMs are probably an evolutionary strategy by which Penicillium molds protect themselves against other microbes and reduce competition with other molds within their surrounding environment. Although it is generally assumed that many PMs are rapidly metabolized by commensal microbes (Abrunhosa et al., 2010; Fuchs et al., 2008; Kiessling et al., 1984), their antimicrobial properties could possibly attenuate microbial detoxifying mechanisms. Furthermore, PMs may also disrupt the normal colonization or maintenance of commensal microbes, which could compromise host protection against pathogenic microbes (Croswell et al., 2009; Stecher et al., 2007) and toxins (Pamer, 2007; Tesh et al., 1994). In support of this hypothesis, enteropathogenic Salmonella Typhimurium was shown to colonize the murine intestine more efficiently when it was not competing with commensal microbiota (Stecher et al., 2007). Additionally, when commensal microbes are disrupted, bacterial toxins from E. coli O157:H7 (Tesh et al., 1994) and endotoxins from Clostridium difficile (Pamer, 2007) can cause systemic pathology.

1.1.3.2. Physical disruption of epithelial surfaces

PMs can also directly disrupt the integrity of epithelial surfaces increasing risk of secondary disease. For example, Maresca et al. (2001) reported a significant reduction in the transepithelial resistance (TER) of HT-29-D4 and Caco-2 cells, which is an indicator of epithelial barrier damage, at OTA concentrations greater than 1 μM. Another study also observed that PAT destabilized epithelial cell membrane cysteine thiol and sulfhydryl groups that compromised HT-29-D4 and Caco-2 cell integrity and reduced TER (Mahfoud et al., 2002). This epithelial barrier damage increases potential uptake of foreign substances such as PMs and pathogenic microbes into the host systemic circulation, which could predispose animals to disease (Maresca et al., 2008).
1.2. *Penicillium* myotoxins (PMs): immunomodulation and immunotoxicity

In general, the paradigm in immunology categorises the immune system into two branches: innate and adaptive immunity for ease of understanding (Murphy et al., 2008), even though some common cells and molecules are involved in both types of immunity. Innate immunity is characterized as the first line of defence during microbial infection after physical barriers have been penetrated. It generally involves immune surveillance, inflammation and immune regulation, all of which are considered non-specific immune responses. Innate immunity is triggered by the recognition of pathogens by humoral and cellular pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMP) with limited specificity and affinity.

Adaptive immunity, on the other hand, recognises and targets pathogens with higher specificity and affinity. In this case, pathogen antigen recognition is mediated by either T-cell receptors on T-lymphocytes, or membrane-bound immunoglobulins on B-lymphocytes and antibodies secreted by plasma cells. Adaptive immunity is most often distinguishable from innate immunity by its ability to induce a memory response that is characterized as having a more rapid response with higher specificity and affinity during subsequent pathogen exposure.

PMs can potentially modulate molecules and cells involved in both innate and adaptive immune responses in many different ways. For example, most of the PMs that will be discussed in this review affect leukocyte cell numbers, which could be due to cell death, and changes in cell proliferation, differentiation and trafficking throughout the body. Furthermore, some of these PMs may also alter the function and expression of cytokines, inflammatory molecules, as well as the expression of immunoglobulins, which will be described in more detail in the following sections. PMs have also been reported to alter the morphological structure and function of lymphoid organs such as thymus, spleen and lymphnodes, which are important for the development, and maintenance, and activation of adaptive immune system.
1.2.1. Ochratoxin A (OTA)

1.2.1.1. Leukocyte number

OTA is a potent immunomodulatory mycotoxin that can reduce circulating numbers of leukocytes leading to leukopenia (Chang et al., 1979; Stoev, 2010). For example, OTA decreased the total number of blood monocytes and lymphocytes in a dose-dependent manner in broiler chickens (Chang et al., 1979), and a significant decrease in the number of blood neutrophils, basophils, and monocytes was also observed in mice fed 5 mg/kg of OTA in feed (Gupta et al., 1979). This decrease in the blood leukocyte number was partially due to the reduced production of leukocytes in lymphoid organs, as OTA targets the primary lymphoid organ, such as bone marrow, thymus and bursa of Fabricius, as well as secondary lymphoid organs, including spleen and lymph node of various species (Boorman et al., 1984; Stoev, 2010; Stoev et al., 2004; Xue et al., 2010). For instance, OTA decreased the number of bone marrow pluripotent stem cells that are required for generating leukocytes such as granulocyte macrophage progenitors, as well as both CD4+ and CD8+ T-cell subpopulations in spleen of mice (Alvarez-Erviti et al., 2005; Boorman et al., 1984). OTA reduces proliferation and viability of these immune cells, leading to the overall decrease in the number of blood and tissue leukocytes (Charoenpornsook et al., 1998). In support of this, OTA prevented the lymphoblastogenesis of porcine and human lymphocytes (Lea et al. 1989; Harvey et al. 1992). Charoenpornsook et al. (1998) also reported that 1.5 μl/ml of OTA inhibited concanavalin A (Con A)-, phytohaemagglutinin (PHA)- and pokeweed mitogen (PWM)-induced bovine peripheral blood mononuclear cell (PBMC) proliferation by 50% (IC50), and similar IC50s have been reported for bovine lymphocytes (Keblys et al., 2004; Stec et al., 2008). Lea et al. (1989) looked more specifically at the effect of OTA on B cell proliferation, which was induced with polyclonal rabbit anti-human IgM antibodies in the presence of bovine B-cell growth factor (BCGF-1); they reported a significant inhibitory effect of OTA (50 μM) on B-cell
proliferation. We have also observed an inhibitory effect of OTA on the proliferation of a bovine macrophage cell line (BoMacs) after the concentration of 5.2 μM (Oh et al., 2012). In contrast, OTA concentration between 0.5 and 20 μM failed to inhibit the proliferation of murine lymphocytes induced by either Con A or lipopolysaccharide (LPS) (Alvarez-Erviti et al., 2005). These contradictory results may be due to the species variation.

1.2.1.2. Inflammatory response and cell death

Several studies have demonstrated the OTA as a potential inducer of inflammatory molecule production by macrophages. Ferrante et al. (2006, 2008) for example, observed a significant increase of pro-inflammatory molecules such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and heat shock protein 72 (hsp72) from murine macrophages and the macrophage cell line J774A. These inflammatory molecules, especially COX-2, stimulate macrophage tumor necrosis factor (TNF)-α production, which is a potent pro-inflammatory cytokine (Al-Anati et al., 2005). These studies have also found a strong positive association between the OTA-induced pro-inflammatory mediators and the activity of nuclear factor (NF)-κB, a transcription factor that stimulate cellular pro-inflammatory response (Al-Anati et al., 2005; Ferrante et al., 2008; Lawrence, 2009). For example, OTA induced p65-NF-κβ protein expression in the macrophage cell line (Al-Anati et al., 2005), and these authors also showed that inhibition of NF-κβ blocked TNF-α release that was induced by OTA from perfused rat liver. Ferrante et al. (2008) later demonstrated that OTA induced the degradation of cytosolic inhibitor of NF-κβ (Iκβ-α), which allows NF-κβ for translocation into nucleus and increased expression of pro-inflammatory molecules and cytokines (Figure 1.2). In addition to this, TNF-α also activates the Jun N-terminal kinase (JNK)-p38 mitogen-activated-protein kinase (MAPK) signaling pathway (Deng et al., 2003), which further enhances the pro-inflammatory response (Elsea et al., 2008), and this was
also previously observed with both canine renal epithelial cells and rat proximal tubular cells (Gekle et al., 2000; Sauvant et al., 2005).

Increased TNF-α production by OTA not only stimulates pro-inflammatory response, but it also affects cell viability. Interaction between TNF-α and membrane-bound TNF-α receptors-1 (TNFR-1) for example, activates TNF receptor-1-associated death domain (TRADD), which then triggers caspase-dependent apoptosis as indicated by a decrease in Bcl:Bax family ratio, the activation of caspases, and the release of mitochondrial protein cytochrome C (Aggarwal et al., 2000; George et al., 2010). It has been proposed that OTA reduces the expression of anti-apoptotic protein Bcl-xL, which results in the release of cytochrome C as well as the activation of caspase-3 and -9. These activated caspases trigger a loss in mitochondrial trans-membrane potential leading to apoptotic cell death (Assaf et al., 2004; Chao et al., 1995; Chen et al., 2007). Activation of the JNK-p38-MAPK pathway by TNF-α further enhances the cell death process (Deng et al., 2003; Gekle et al., 2000). Lastly, OTA was also shown to induce apoptosis, independent of interaction between TNF-α and TNFR-1 in hepatocytes, which appears to be due to intracellular oxidative stress as indicated by increased production of H₂O₂, which then triggers apoptosis (Buttke and Sandstrom, 1994; Essid et al., 2012).

1.2.1.3. Effect on adaptive immunity

In addition to stimulating the pro-inflammatory response and death of leukocytes, OTA also modulates other immune cell functions, specifically down-regulating the cell-mediated immune response (CMIR). Harvey et al. (1992) reported that feeding 2.5 mg of OTA to growing gilts during a one-month period decreased the CMIR, including macrophage phagocytic activity, cutaneous basophil hypersensitivity, as well as delay-type hypersensitivity response.
The decreased CMIR may be partially attributed to the decreased total number of T-cells since OTA inhibits T-cell proliferation and viability (Alvarez-Erviti et al., 2005; Chang et al., 1979; Keblys et al., 2004). However, several other studies also discussed that the selective suppression of the CMIR by OTA exposure might have also occurred via the inhibition of both type-I and type-II interferon (IFN) production (Luster et al., 1987; Xue et al., 2010). The type-I IFNs, IFN-α and IFN-β, are potent activators of CMIR, and their suppression decreases NK cell anti-viral and anti-tumor activity (Swann et al., 2007). Type-2 IFN-γ is a crucial cytokine for promoting cytotoxic T-cells (CTLs) and macrophage activation (Schroder et al., 2004; Wigginton et al., 2001). Luster et al. (1987) reported that oral exposure of mice to OTA at the doses between 3.4 to 13.4 mg/kg decreased the serum concentration of total IFNs in a dose-dependent manner. They also observed selective inhibition of natural killer (NK) cell anti-tumor activity against murine mastocytoma P815 tumor cells, but not anti-tumor activity of either blood CTLs or macrophages. This finding indicates that OTA may specifically inhibit the production of type-I IFNs in mice. However, a much lower concentration of 0.25 mg/kg OTA, when co-exposed with T-2 toxin, led to a significant decrease in serum IFN-γ concentration as well as a slight reduction in the splenic gene expression of IFN-γ in broiler chickens (Xue et al., 2010). Furthermore, in vitro exposure of OTA significantly inhibited not only the cytotoxic activity of NK cells but also the cytotoxic activity of CTLs against mouse lymphoma Yac-1 target cells (Alvarez-Erviti et al., 2005). Oral administration of 0.05-0.45 mg/kg of OTA to male rats also decreased the cytotoxic activity of CTL and splenocytes against sheep red blood cells (sRBC) (Alvarez et al., 2004). Based on these studies, it can be suggested that OTA suppresses not only type-I IFN, but may also suppress the IFN-γ that is important for stimulating the cytotoxic activity of CTL (Wigginton et al., 2001).
Some studies have reported that OTA also modulates the antibody-mediated immune response (AbMIR). For example, intraperitoneal injection of 1 μg/kg of OTA significantly decreased sRBC-specific IgG and IgM responses by 90% in mice (Creppy et al., 1983). This decrease may be attributed to a decrease in the total number of B-cells, since OTA was shown to decrease lymphocyte numbers by inhibiting cell proliferation as discussed earlier (Lea et al., 1989). In contrast to the previous study, the oral administration of 2.5 mg/kg OTA to pigs did not affect total immunoglobulin concentrations, or the specific AbMIR to chicken RBCs (Harvey et al., 1992). It is possible that intraperitoneal injection of OTA, used in the Creppy et al. (1983) murine study, might have bypassed microbial and hepatic metabolism, which were previously shown to detoxify mycotoxins (Reddy et al., 1982; Thust and Kneist, 1979).

1.2.2. Citrinin (CIT)

1.2.2.1. Leukocyte number

Similar to OTA, CIT has significant effects on leukocyte numbers. For example, CIT exposure reduced the proliferation of porcine and bovine T-lymphocytes, in vitro (Keblys et al., 2004; Stec et al., 2008). The CIT doses of 5 or 10 mg/kg BW via oral gavage also decreased the number of murine splenic macrophages, CD8+ CTLs and CD4+ T-helper cells, while the total number of B cells was reduced in intestinal intra-epithelium, lamina propria and Peyer’s patches of BALB/c mice (Islam et al., 2012). In contrast, these authors also observed that the CIT exposure increased the number of CTLs, T-helper cells, and CD4+CD25+Foxp3+ regulatory T-cells in mesenteric lymph nodes, as well as intestinal CTLs in the intra-epithelium, lamina propria and Peyer’s patches of mice. Another in vivo study reported similar results, where they observed T-cell blastogenesis was stimulated in the spleens of the mice that received CIT at the doses higher than 0.12 mg/kg of ip injection (Reddy et al., 1988). Islam et al. (2012) suggested that the increase in T-cell population
may be due to CIT acting as a T-cell-specific mitogen, activating the NF-κβ or MAPK. However, they also stated that CIT could also stimulate the migration of T-cells from spleen to other immune tissues, which would explain the increased T-cell numbers that they observed in most secondary lymphoid organs, and reduced numbers of T-cells in the spleen. This seems to be a suitable explanation for the *in vivo* studies; however, it does not explain the suppressed T-cell proliferation observed *in vitro*, since CIT should have induced the T-cell proliferation if CIT was indeed a T-cell mitogen. Therefore, there seems to be more complex interaction among T-cells and other cell types when animals are exposed to CIT as compared to *in vitro* exposure. In support of this, Dziarski (1985) previously reported a higher murine lymphocyte mitogenic response *in vivo* than *in vitro* following exposure to various types of mitogens. Furthermore, the effect observed in the *in vivo* studies may not be due to CIT in its original form, but rather the effect of its metabolites. CIT is metabolized in the liver and possibly other organs, and these metabolites may have a different potency and toxic effects in animals (Reddy et al., 1982; Thust and Kneist, 1979). It is also possible that the mitogenic or inhibitory response is dose-dependent, where a low concentration of CIT could have resulted in hormetric effect *in vivo*. Hormesis is a biphasic concentration-response relationship manifesting as a stimulating response at low concentrations, followed by an inhibitory effect at high concentrations (Calabrese and Baldwin, 2003).

1.2.2.2. Oxidative stress and cell death

Regardless of potential mitogenic activity of CIT on lymphocyte proliferation, CIT can also decrease total leukocyte numbers by inducing cell death. Similar to OTA, CIT triggers the apoptosis of leukocytes by inducing caspase pathways (Kumar et al., 2011), and this is probably initiated by increased intracellular oxidative stress.
CIT induces oxidative stress by decreasing the level of intracellular anti-oxidant, glutathione (GSH) (Kumar et al., 2011), and this in turn reduces other GSH-dependent anti-oxidant enzymes such as GSH reductase (GSSG) and trans-hydrogenases (Ribeiro et al., 1997). Intracellular glutathione peroxidase (GSH-Px) functions as an anti-oxidant enzyme that inhibits the production of reactive oxygen species (ROS) molecules, such as superoxide anion and hydrogen peroxide (Papp et al., 2012). Oxidative stress subsequently stimulates the production of superoxide anion and ROS via the mitochondrial respiratory chain pathway (Chagas et al., 1992), while at the same time arrests the G0 to G1 phase of cell cycle, reduces Bcl-2/Bax activity ratio, and induces cytochrome C release from mitochondria; all of which eventually stimulate and sensitize cells to the p53-mediated caspase-3 and -9 apoptotic death signal (Basu and Haldar, 1998; Islam et al., 2012; Kumar et al., 2011). CIT has also been shown to reduce the gene and protein expression of the major inflammatory cytokines interleukin (IL)-1β, IL-6 and TNF-α in human Mono-Mac-6 cells (Johannessen et al., 2005) and murine macrophages (Islam et al., 2012). In contrast, Liu et al. (2010) observed CIT inhibited the Janus kinase (JAK)/signal transducers and activators of transcription (STAT)-1α and NF-κB signaling pathways, leading to the inhibition of iNOS and nitric oxide (NO) production in a glomerular mesangial cell line in response to LPS and IFN-γ exposure. Therefore, CIT appears to induce leukocyte oxidative stress (Kumar et al., 2011; Ribeiro et al., 1997), independent of NO production, which in turn activates the intrinsic apoptosis pathway that involves stimulation of p53-mediated caspase-3 and caspase-9 (Chan, 2007; Islam et al., 2012; Kumar et al., 2011).

1.2.2.3. Effect on adaptive immunity

CIT appears to affect the balance of T helper cell type (Th)1 and Th2 cytokines that support CMIR and AbMIR, respectively. An in vitro exposure study for example, reported that CIT
suppressed both IFN-γ and IL-4 production from CD3- and CD28-stimulated human blood mononuclear cells in a dose-dependent manner (Wichmann et al., 2002); the IC50 for cytokine production was 21.6 μg/ml for IL-4, however, it was only 8.3 μg/ml for IFN-γ production. This suggests CIT more severely affects IFN-γ production than IL-4 production thereby favouring AbMIR over CMIR at low exposure concentrations. Taken together, the suppression of iNOS and IFN-γ production by CIT has the potential to compromise host defence against intracellular pathogens (Liu et al., 2010). In support of this, Herzog-Soares and Freire (2004) demonstrated that when murine macrophages were pre-exposed to 10 μg/ml of CIT, the infectivity of the cells by *Toxoplasma gondii* tachyzoites (*T. gondii*) increased by 18%, and also enhanced the replication of the tachyzoites within the cells.

At higher concentrations, CIT also appears to suppress AbMIR, since CIT was shown to decrease total B-cell number as well as IL-4 production at higher concentrations (Islam et al., 2012; Wichmann et al., 2002). Furthermore, 2.5 mg/kg of CIT ip injection decreased both sRBC-specific antibody and complement activity by 75-87.5% and 87.5-93.8%, respectively, in albino mice (Carvalho et al., 2005). Similar results were also observed in Wistar rats that were fed 10 mg/kg of CIT when sRBC- and ovalbumin-specific antibody responses were measured (Singh et al., 2011).

1.2.3. Patulin (PAT)

1.2.3.1. Leukocyte number

PAT is a potent immunotoxin that can severely alter the number of leukocytes in blood and tissues, and modulate host immune function. *In vitro* studies have demonstrated that PAT reduces the proliferation and viability of leukocytes, specifically T-cells and macrophages (Keblys et al., 2004; Oh et al., 2012; Sorenson et al., 1985). For example, Keblys et al. (2004) observed dose-
dependent decrease in the proliferation of blood lymphocytes when porcine T-cells were exposed to PAT *in vitro*.

*In vivo* studies have also demonstrated similar effects on the number of blood leukocytes from mice exposed orally to PAT, by both acute and chronic exposure. Escoula et al. (1988) for example, reported a significant decrease in total blood lymphocyte numbers in Swiss mice fed 10 mg/kg of PAT for 5 to 10 days. A similar observation was also reported with B6C3F mice that consumed PAT at concentrations greater than 1.28 mg/kg for 28 days, where PAT decreased the number of total blood leukocytes by 30% (Llewellyn et al., 1998). These authors also reported that PAT increased the number of certain subsets of immune cells at a predicted human exposure level of 0.08 – 2.56mg/kg. For example, PAT after a 28 day exposure period, increased the number of splenic CTLs, NK cells and monocytes by 50%, 30% and 24%, respectively, while the number of CD3+T-cells, CD4+T-cells, and MAC-3+ monocytes remained unchanged (Escoula et al., 1988; Llewellyn et al., 1998). Similar to the 28-day chronic exposure study, acute 4-day exposure of mice to 10 mg/kg PAT via stomach tubing, and intraperitoneal injection of 2.5 mg/kg PAT in rabbit did not affect the number of either splenic neutrophils or CTLs, but increased the number of splenic T-cells in both mice and rabbit even though the mitogenic response of T-cells and the number of splenic B cells were suppressed (Escoula et al., 1988). Furthermore, another *in vivo* study reported that 0.1 mg/kg of PAT for 90 days significantly increased apoptosis of rat thymic dendritic cells (DCs) that are required for normal T-cell development (Ozsoy et al., 2008).

1.2.3.2. Oxidative stress and cell death

In addition to the above reported cytotoxic effects of PAT on rat thymic DCs (Ozsoy et al., 2008), *in vitro* studies have also shown PAT-induced cytotoxicity to rat alveolar macrophages (Sorenson et al., 1985), and a bovine macrophage cell line (BoMacs) (Oh et al., 2012). Similar to
OTA and CIT, PAT appears to induce p53-mediated apoptotic cell death triggered by caspase-3 and -9 pathways, which was observed in both murine skin cells (Saxena et al., 2009), and human leukemia HL60 cells (Wu et al., 2008). PAT acted as a strong inhibitor of inflammatory cytokines, suppressing the production of IL-1β, IL-6 as well as TNF-α and TNF-β from human mononuclear cells and T-cells (Luft et al., 2008). However, PAT also induced ROS production leading to intracellular oxidative stress, which then stimulated the caspase pathways to induce cell apoptosis (Liu et al., 2007; Papp et al., 2012).

The mechanism by which PAT induces oxidative stress appears to resemble that of CIT, where both mycotoxins have been shown to inhibit anti-oxidant enzymes, specifically targeting the intracellular GSH-dependent enzymes, such as GSSG (Luft et al., 2008; Papp et al., 2012; Ribeiro et al., 1997). Although it is still unclear for CIT, it appears that PAT destabilizes the disulfide bonding of cysteine within GSH (Papp et al., 2012; Schütze et al., 2010). PAT-induced ROS production damages DNA arresting the cell cycle, and this would eventually initiate caspase apoptotic death pathway by inducing Bax pro-apoptotic protein expression as well as p21 and p53 (Saxena et al., 2009; Wu et al., 2008), subsequently stimulating the apoptosis of leukocytes.

1.2.3.3. Effect on adaptive immunity

Intracellular GSH levels have also been explored as a marker to determine the polarization of DCs, with reduced GSH levels being a marker of DCs of the Th2 phenotype that support AbMIR (Peterson et al., 1998). Studies have reported that PAT decreased intracellular GSH levels in human PBMCs, T-cells as well as murine DCs (Luft et al., 2008; Schütze et al., 2010). These cells, at the same time, expressed a Th2 dominant phenotype in terms of their cytokine production. For example, studies have shown that PAT decreased the production of both IFN-γ and IL-12. These cytokines are crucial for promoting a Th1 response, and stimulate the CMIR. Impaired production
of these cytokines could increase host susceptibility to intracellular pathogens, such as \textit{T. gondii} (Hunter et al., 1994) or \textit{Mycobacterium} (Wagner et al., 2002). In support of this, PAT at a concentration greater than 0.5 $\mu$g/ml in media was shown to severely suppress the phagocytic and microbicidal activity of murine peritoneal macrophages to yeast \textit{Saccharomyces cerevisiae} (Bourdiol et al., 1990).

In contrast to its effect on the CMIR, PAT had less effect on the expression of Th2 cytokines, such as IL-5 and IL-13, from murine splenocytes and mediastinal lymph node cells (Schütze et al., 2010). The production of IL-4 by human PBMCs on the other hand, was inhibited at 0.050 $\mu$g/ml of PAT (Luft et al., 2008), but this study also observed greater suppression of IFN-$\gamma$ production as compared to that of IL-4. In fact, a low concentration of 0.003 $\mu$g/ml PAT, rather increased human PBMC IL-4 production, but suppressed IFN-$\gamma$ production suggesting a possible quadratic effect of PAT on IL-4 production (Wichmann et al., 2002). PAT also suppressed the production of other cytokines, including IL-2 and IL-10, all of which are important for the survival and regulation of leukocyte function (Colic et al., 2003; Dooms et al., 2004).

Similar to CIT, PAT at high doses may also suppress the AbMIR by down-regulating plasma cell (PC) antibody production. For example, intake of 10 mg/kg of PAT by oral gavage and intraperitoneal administration of 2.5 mg/kg of PAT dramatically decreased total serum levels of IgG, IgA and IgM without altering levels of serum albumin and complement component C3 (Escoula et al., 1988b). The reduction of these immunoglobulins may be attributed to B-cell apoptosis. Other studies using a lower dose of PAT exposure reported that PAT did not necessarily decrease immunoglobulin production. In fact, 28 days oral exposure to 0.08 – 2.56 mg/kg of PAT, unlike CIT, did not alter the serum IgM response to sRBC in B6C3F1 (Carvalho et al., 2005; Llewellyn et al., 1998). Furthermore, the oral and nasal exposure to 5 – 20 $\mu$g of PAT also increased
ovalbumin (OVA)-specific IgE and IgG1 in BALF/C mice without altering IgG2a levels (Schütze et al., 2010). The increased IgG1/IgG2a is also an indication of a Th2 response, suggesting that PAT shifts the immune response toward Th2 (Schütze et al., 2010). T-cells on the other hand, appear to remain viable during PAT exposure, even though the capacity to produce certain cytokines, especially those that are important for CMIR, is severely compromised (Colic et al., 2003; Dooms et al., 2004; Wichmann et al., 2002).

1.2.4. Penicillic acid (PA)

1.2.4.1. Leukocyte number

There are only few studies available on the subject of PA immunotoxicity. PA may not necessarily alter immune function, yet it was shown to reduce the proliferation and viability of immune cells, decreasing the overall number of immune cells in vivo. For example, feeding a diet containing 2 mg/kg of PA to broiler chicks led to a severe decrease in the weight of both primary (thymus and bursa of Fabricus) and secondary lymphoid organs (spleen), and this effect worsened during co-exposure with OTA (Stoev et al., 2004). The reduction in lymphoid organ size may be due to suppressed proliferation of immune cells such as macrophages and lymphocytes rather than the reduced viability, because a much higher PA concentration is required to induce cell death (Keblys et al., 2004; Oh et al., 2012; Stoev et al., 2004). Stoev et al. (2004) also observed a dose-dependent decrease in the proliferation of poultry PBMCs, but they were unable to reproduce these results using human PBMCs in a later study (Stoev et al., 2009). The IC50s for PA based on cell proliferation have been determined to be 18.0 and 30.0 μM for porcine T-cells and bovine macrophages, respectively (Keblys et al., 2004; Oh et al., 2012).
1.2.4.2. Cell death via necrosis

PA can also decrease the viability of immune cells, yet this occurs at much higher concentrations. Sorenson and Simpson (1986) for example, reported that concentrations of PA greater than 1.0 mM caused significant cytotoxicity to rat alveolar macrophages after 2 hours of exposure. PA concentrations greater than 160 μM also significantly decreased the viability of bovine macrophages when exposed for 24 hours, and the LC50 was determined to be 176.0 μM (Oh et al., 2012).

In contrast to the PMs discussed earlier, the cytotoxicity induced by PA does not occur via apoptosis but rather necrosis, whereby PA reduces cell membrane integrity, leading to cytolysis of immune cells (Bando et al., 2003). Actually, concentrations of PA between 100 and 200 μM effectively blocked the activity and self-activation of caspase-3, -8, and -9 in a lymphoma cell line, and the inhibition of these caspase pathways resulted in the decreased DNA fragmentation and apoptotic cell bodies. However, these concentrations at the same time caused unfavourable cell necrosis that reduced the overall cell viability (Bando et al., 2003).

PA could also affect other parameters of immune system, even though this has not widely been studied. Sorenson and Simpson (1986) reported the reduced phagocytic capacity along with the suppressed RNA and protein synthesis in rat alveolar macrophages that were treated with 0.3 mM PA for 2 hours. The metabolic rate of the human PBMC, on the other hand, was induced by PA (Stoev et al., 2009). Changes in RNA and protein synthesis, as well as metabolic rate, could potentially affect the production of anti-microbial molecules produced by immune cells, however, we are unaware of any studies that have been carried out to assess the potential effect of PA related to these functional aspects.
1.2.5. *Mycophenolic acid* (MPA)

MPA is a well-known potent immunosuppressive PM that has been effectively used to prevent organ rejection following transplantation (Borrows et al., 2006), as well as treat inflammatory disorders such as arteriosclerosis and rheumatoid arthritis (Cohn et al., 1999; Mohacsi et al., 1997). For these reasons, MPA and its derivative mycophenolate mofetil (MMF) are by far the most widely studied PMs in terms of their immunomodulatory properties. Both functional and transcriptomic studies have revealed that MPA most frequently mediates alterations in cell proliferation, viability as well as the expression of co-receptors required for migration and activation of immune cells (Dubus et al., 2002; Dun et al., 2013; Hauser et al., 1997; Mezger et al., 2008).

1.2.5.1. Innate immune cell proliferation

The effect of MPA on cell proliferation and viability has been thoroughly studied in human medicine (Borrows et al., 2006; Cohn et al., 1999). A study performed by Borrow and colleagues (2006) reported that when a high oral dose of MPA, up to 1.95 g/day, was given to patients following renal transplantation, there was a significant decrease in the number of blood leukocytes compared to the patients who received a low dose of MPA post transplantation. MPA and MMF likely cause a decrease in the proliferation of leukocytes resulting in reduced leukocyte numbers (Dubus et al., 2002; Mezger et al., 2008; Ohata et al., 2011). For, example, MPA and MMF, at clinically effective concentrations between 0.6 and 10 μM in media, have been shown to inhibit the proliferation of rat and human mesangial cells without inducing cellular necrosis or apoptosis (Dubus et al., 2002; Hauser et al., 1997). Other studies have demonstrated that MPA also inhibited the proliferation of different immune cell types including bovine macrophages, human monocyte-derived dendritic cells (mDC), polymorphonuclear cells and NK cells without causing apoptosis.
or necrosis (Mezger et al., 2008; Oh et al., 2012; Ohata et al., 2011). The decreased number of these innate immune cells may partially explain why there was reduced rejection in transplant recipients following treatment with MPA.

1.2.5.2. Inhibition of NK cell activation

NK cells are important mediators of the immune response and that limit the growth of tumors and spread of microbial infection (Ohata et al., 2011), and also contribute to allograft rejection during the first month of post-transplantation (Eissens et al., 2010; Kroemer et al., 2008). For these reasons, NK cells are one of the innate immune cell groups that have most widely been studied in the context of MPA immunosuppression. Many studies have shown that MPA decreases the activity of NK cells by inhibiting their activation (Ohata et al., 2011; Weigel et al., 2002). For example, when human NK cells were incubated with leukemic cells and exposed to 10 µg/ml of MPA in vitro for 7 days, there was a significant decrease in their activation markers, NKG2D, NKp30, NKp44 and NKp46 (Ohata et al., 2011). These NK cells also had reduced cytotoxic activity to leukemic cells, K562 and Daudi (Eissens et al., 2010; Ohata et al., 2011) Eissen et al. 2010 also reported that MPA decreased the shift in NK cell receptor repertoire, such as NKG2A, which might have partially contributed in the decreased cytotoxic activity of NK cells against K562 (Eissens et al., 2010). The decrease in the activation markers from the studies indicate that MPA has inhibitory effects on both the maturation and activation of NK cells, thereby reducing their cytotoxic activity. This is one of reasons why MPA has been used as an effective post-transplantation drug.

1.2.5.3. Changes in the cellular surface protein expression on dendritic cells (DCs)

DCs are important regulators of the immune system as they are considered to be key mediators of naïve lymphocyte activation (Novak et al., 2010). These cells are highly susceptible to
immunomodulation by MPA. It is thought that MPA affects the expression of membrane proteins on DCs that are required for the activation and function of the host immune response (Cicinnati et al., 2009; Hauser et al., 1997). For example, one study demonstrated that in vivo exposure of MPA reduced the trafficking of human blood myeloid DCs to lymph nodes by down-regulating chemokine receptor (CCR)-7, but increased the migration of these cells to sites of inflammation by up-regulating CCR-1 expression (Cicinnati et al., 2009). MPA also simultaneously induced the expression of endothelial VCAM-1 and E-selectin, both of which are required for the transmigration of leukocytes across endothelial cells during inflammation (Hauser et al., 1997). Therefore, these studies suggest that MPA stimulates the recruitment of mDCs towards sites of inflammation.

MPA also inhibits the maturation of DCs thereby limiting their ability to stimulate other immune mediators, such as T and B cells (Cicinnati et al., 2009; Hauser et al., 1997). The surface proteins on DCs play an essential role in lymphocyte activation by acting as co-receptors during antigen presentation to T and B cells (Novak et al., 2010). Therefore, the down-regulation of the co-receptors would lead to incomplete activation of lymphocytes, and reduce the function of lymphocytes (Daniel et al., 1997; Vogel et al., 2013). For example, the study performed by Cicinnati and colleagues (2009), demonstrated that MPA inhibited the maturation of human mDCs by impairing TLR-3 ligation to polyinosinic-polycytidylic acid, a synthetic double stranded RNA used as a viral antigen to activate innate cells. Other in vitro studies also reported the down-regulation of human mDC maturation markers, such as CD80 and CD86, as well as other co-stimulatory and adhesion molecules, CD1a, CD40, CD54, CD83 and CD 205 (Colic et al., 2003; Mezger et al., 2008; Wadia et al., 2009). The in vitro exposure of MMF also down-regulated the expression of co-receptors, CD40, CD80/86, and ICAM-1, on murine DCs (Mehling et al., 2000).
DCs lacking in the above co-receptors would not be able to efficiently activate lymphocytes, and therefore, would affect the maturation and function of both B- and T-cells (Daniel et al., 1997; Vogel et al., 2013; Wykes and Macpherson, 2000). The absence of CD40 signal from DCs for example, impairs the proliferation as well as maturation of B-cells (Wadia et al., 2009; Wykes and Macpherson, 2000). Additionally, incomplete activation of T-cells either stimulates apoptosis of the T-cells, or favors their differentiation into T-reg cells (Daniel et al., 1997; Vogel et al., 2013). In support of this, human DCs, when pre-treated with MPA, stimulated the differentiation of naïve T-cells to T-reg cells with increased production of IL-5 and anti-inflammatory cytokines, IL-10 and TGF-β (Colic et al., 2003; Lagaraine et al., 2008). IL-10 and TGF-β are strong immunosuppressive cytokines that are required for the differentiation of T-reg cells (Taylor et al., 2006). Interestingly, direct exposure of naive and memory human T-cells with 1 µM MPA also induced the differentiation to T-reg cells, as indicated by the increased expression of the T-reg cell markers, Foxp3, CTLA4 and PD-1 (Deng et al., 2012; He et al., 2011). Based on these studies, it is speculated that MPA increases the T-reg cell population, via DC dependent and independent signals. Since T-reg cells have strong immunosuppressive phenotypes (Sakaguchi, 2004), an increase in their number likely leads to immunosuppression, and make MPA an effective compound for preventing allograft rejection (Borrows et al., 2006; Mohacsi et al., 1997).

1.2.5.4. Proliferation and viability of T-cells

It appears that the immunosuppressive effect of MPA may occur not only through the upregulation of T-reg cell differentiation but also by reducing the number of T-cells (Chapuis et al., 2000; Keblys et al., 2004; Quéméneur et al., 2002). For example, a patient treated with 2 g/day of MPA for a year following organ transplantation had reduced numbers of total T-cells in their
peripheral blood (Weigel et al., 2002). This decrease in lymphocyte number is partially due to MPA’s ability to inhibit cell proliferation as well as its cytotoxicity to T-cells (Chapuis et al., 2000).

It has been proposed that the selective effect of MPA on proliferation and viability of activated T-cells is closely linked to down-regulation of type II inosine monophosphate dehydrogenase (IMPDH-II) activity, which inhibits *de-novo* purine biosynthesis (Dun et al., 2013; Hauser et al., 1997; Nagai et al., 1992). There are two isotypes of IMPDH, type I and II. Both isotypes have similar enzymatic function for purine biosynthesis, yet they are differentially expressed in different cell types. For example, Nagai and colleagues (1992) demonstrated that activated T-cells and HL60 leukemic cells predominantly express type II IMPDH, while type I IMPDH was more constitutively conserved across most other cell types. Regardless of their similar functionality, the activity of type I IMPDH cannot be substituted for type II IMPDH (Gu et al., 2000; Jain et al., 2004). Activated T-cells and leukemic cells rely heavily on type II IMPDH for GTP synthesis, while naïve T-cells and other cell types, such as macrophages or epithelial cells, actively utilize type I IMPDH (Karnell et al., 2011; Nagai et al., 1992). MPA was shown to specifically down-regulate type II IMPDH and had no effect on Type I IMPDH (Hauser et al., 1997; Nagai et al., 1992), and this would allow MPA to selectively target the proliferation and viability of activated lymphocytes or other leukemic cells. Inhibited purine biosynthesis compromises the cell’s ability to synthesize GTP/GDP, which is essential for normal immune cell functions such as differentiation, proliferation and apoptosis (Nagai et al., 1992; Yalowitz and Jayaram, 2000). The decreased expression of GTP following MPA treatment partially led to the reduction of the ERK 1/2 and MAPK pathways, which could reduce the all viability by activating capase apoptotic death signals (Allan et al., 2003; Ha et al., 2006). For example, MPA induced the cell death of the MOLT-4 T-lymphocytic cell line, as well as PHA-activated CD4+ T-cells, but not resting naïve T-
cells (Chapuis et al., 2000; Cohn et al., 1999). Other studies have reported that MPA induced apoptosis in the Jurkat human T-cell line and gastric cancer cell line, as indicated by increased caspase-3 and -9 activity (Choi et al., 2013; Dun et al., 2013).

The depletion of cellular GTP/GDP not only trigger apoptosis, but also causes necrotic cell death in some immune cell types (Chaigne-Delalande et al., 2008; Guidicelli et al., 2009). Chaigne-Delalande and colleagues (2008), demonstrated that MPA caused cytotoxicity to B- and T-lymphoma cell lines as well as mitogen-activated human PBLs by necrosis or caspase-dependent apoptotic pathways. MPA did not necessarily induce either apoptosis or necrosis to naïve PBLs, but rather cause them to become sensitized to MPA-mediated necrosis upon activation (Chaigne-Delalande et al., 2008). Necrotic cell death was also demonstrated using the Jurkat^{DoxR} and CEM^{DoxR} lymphocytic leukemia cell lines, which are resistant to doxorubicin-induced apoptosis (Guidicelli et al., 2009). Therefore, MPA specifically targets activated lymphocytes that heavily rely on type II IMPDH activity for GTP/GDP synthesis, causing either apoptosis or necrosis.

1.2.5.5. Effect on B-cell activation

Some studies have also investigated the potential effect of MPA and MMF on the activities of B-cells (Eickenberg et al., 2012; Karnell et al., 2011). Weigel and colleagues, 2002, reported that MMF treatment resulted in almost a 50% reduction in B-cell numbers in patients who received MMF for a year (Weigel et al., 2002). The study also reported that these B-cells from MMF-treated patients were lacking the B-cell activation marker CD69. Karnell et al. (2011) also noted that in vitro MPA exposure to concentrations between 1.0 and 3.0 μM selectively impaired the early activation of human B-cells. Similarly, another study reported significant decreases in the activation as well as differentiation of human B-cells when these cells were exposed to 5 μM MPA in vitro (Eickenberg et al., 2012).
The decreased activation and differentiation of B-cells would decrease the number of plasma cells (PCs), terminally differentiated effector B cells that have a high capacity for producing antibodies (Murphy et al., 2008), subsequently decreasing total antibody levels. In support of this, Eickenberg et al., 2012, reported that the patients who received an oral dose of MMF between 0.3 and 0.5 µM had significantly lower serum IgG concentrations compared to those from other treatment groups. In addition to this, Karnell et al. also observed that in vitro exposure to MMF significantly decreased the production of IgG, IgM and IgA from PCs.

Regardless of the significant effect of MPA on B-cell activation and differentiation (Eickenberg et al., 2012; Karnell et al., 2011; Weigel et al., 2002), MPA does not seem to affect cell viability and/or the responsiveness of terminally differentiated human plasma cells (PCs). Karnell et al. 2011 discussed that the selective effect of MPA on B-cells but not PCs is probably due to the differential utilization of IMPDH enzymes. B cells, like activated T-cells, are predominantly dependent on type II IMPDH activity for GTP/GDP, while terminally differentiated PCs use type I IMPDH (Karnell et al., 2011).

1.2.6. Roquefortine C (RQC)

1.2.6.1. Immunomodulatory effect of RQC

RQC is a common metabolite of P. roqueforti, which is required in the making of blue cheese for human consumption (Auerbach et al., 1998). In terms of the toxicity, RQC is probably the least toxic PM discussed in this review. However, studies have reported various toxicological signs such as paralysis, trembling and diarrhea are associated with RQC exposure to both monogastric (Rand et al., 2005; Scott and Kennedy, 1976; Wagener et al., 1980) and ruminant species (Häggblom et al., 1990), yet very few studies are available on its potential immunotoxicity (Keblys et al., 2004; Rand et al., 2005).
Similar to the other PMs, RQC can also inhibit the proliferation of lymphocytes. For example, in vitro RQC exposure suppressed the proliferation of porcine lymphocytes, but to a much lesser extent when compared to other PMs, such as OTA, CIT or PAT (Keblys et al., 2004). In addition to this, Rand et al. 2005 observed that the intra-tracheal instillation of RQC that was dissolved in saline solution resulted in a significant increase in the numbers of macrophages and neutrophils in bronchoalveolar lavage fluid (BALF) of mice after 6 hours of the RQC exposure (Rand et al., 2005). There was also a significant increase in BALF inflammatory molecules including MIP-2 and TNF-α. Rand and colleagues in their later study suggest that fungal compounds, such as RQC, with low molecular weight may elevate inflammatory gene transcription in alveolar macrophages (Rand et al., 2011), inducing pro-inflammatory molecules, such as MIP-2 and TNF-α (Rand et al., 2005). Based from these studies, it can be speculated that RQC irritated the lung tissues to induce the infiltration of immune complexes, such as neutrophils and macrophages, and the elevation of other inflammatory mediators, such as TNF-α, in the mice BALF (Anderson et al., 2012; Rand et al., 2005). This however, would require more evidence to support the speculation at this point.

Considering that high concentrations of RQC (25 mg/kg) was shown to cause various toxicological signs in ruminant species (Häggblom et al., 1990) and the concentration of RQC in animal silage reported ranged from 0.4 to 17 mg/kg (Auerbach et al., 1998; Mansfield et al., 2008), it would be important to investigate the immunotoxicity of RQC.

1.3. Epigenetic changes and its relation to inflammatory response

Epigenetics are heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence and these tend to follow environmental cues (Delcuve et al., 2009). These changes are often regulated through DNA and/or histone covalent modifications, such as methylation or acetylation (Fuks et al., 2003; Rice and Allis, 2001), and are
crucial for optimal cellular activity. The regulation of epigenetic modifications is important for the proper functioning of immune cells (Bowdridge and Gause, 2010). For example, M2-macrophage specific genes were regulated by reciprocal changes in the methylation status of lysine 4 and 27 on histone H3 (H3K4 and H3K27), both of which are regulated by epigenetic enzymes (Biel et al., 2005).

As previously discussed, the exposure of *Penicillium* mycotoxins could lead to cellular and functional changes of the immune response (Oh et al., 2012; Sorenson and Simpson, 1986; Sorenson et al., 1985). It has been proposed that some of these functional changes may be influenced by alterations in epigenetic patterns and the activities of the enzymes regulating them (Marin-Kuan et al., 2007, 2008). Given this, it seems reasonable that exposure to mycotoxins may also alter the functioning of immune cells through epigenetic modifications (Bowdridge and Gause, 2010; Marin-Kuan et al., 2008). In support of this, Marin-Kuan et al. (2007) reported that chronic OTA exposure resulted in an increased concentration of histone deacetylase (HDAC)-3 protein in mice kidneys. HDAC-3 is a class-1 histone deacetylase (HDAC) that regulates cell functions by deacetylating lysine residues in histone 3 and/or 4. The class-1 HDACs stimulate inflammatory activities of cells by interacting with signalling molecules involved in NF-κβ transcriptional pathway (Figure 1.2; Ashburner et al., 2001; Chen et al., 2001; Rahman et al., 2004). The inhibition of HDACs suppresses proteasome activity, and reduces the degradation iκβ-α (Al-Anati et al., 2005; Place et al., 2005), nuclear translocation and overall activity of NF-κβ (Ferrante et al., 2008). OTA was shown to reduce the level of cytosolic iκβ-α expression, and this in turn, elevated of nuclear p65- NF-κβ expression in rat liver (Al-Anati et al., 2005). Additionally, decreases in the activity of HDACs demonstrated impairment of NF-κβ signalling pathways and decreased inflammatory response in human myeloid dendritic cells (Song et al., 2011; Zhu et al., 2010). All
these studies indicate that OTA induces pro-inflammatory response by up-regulating the HDAC-3 activity, which activates NF-κβ inflammatory pathways (Al-Anati et al., 2005; Ferrante et al., 2008; Lawrence, 2009; Marin-Kuan et al., 2007).

Recently, many studies have shown that HDAC-3 can alter the stability of the STAT-3 dimer (Sica and Bronte, 2007; Yuan et al., 2005). The STAT-3 dimer functions as a transcription molecule for differentiating M2 macrophages that exhibit anti-inflammatory phenotypes (Mullican et al., 2011; Stout et al., 2005). Upon the stimulation of macrophages with anti-inflammatory cytokines such as IL-4, the cytoplasmic portion of the IL-4 receptor acts as a kinase to phosphorylate cytoplasmic STAT-3 (Choi et al., 2013). The phosphorylated STAT-3 subsequently translocates into the nucleus with the help of importin-3 (Liu et al., 2005). These phosphorylated STATs then spontaneously bind to each other to form STAT-3 dimers (Liu et al., 2005). However, Type-1 HDACs such as HDAC-3, deacetylate lysine$^{685}$ of STAT-3 to destabilize its dimerization, which prevents M2 macrophage differentiation (Yuan et al., 2005). In support of this, Mullican et al. (2011) reported that the macrophages lacking HDAC-3 are hyper-responsive to IL-4 stimulation, resulting in IL-4-induced alternative activation of macrophages in both in vivo and in vitro. From these studies, it is speculated that the increased HDAC-3 activity by OTA could prevent the differentiation of macrophages into M2 macrophages (Stout et al., 2005).

Other epigenetic enzymes, such as DNA methyltransferases, histone demethylase and histone acetylase (Bmi-1), also play an important role in regulating cellular activities (Fatemi et al., 2002; Subkhankulova et al., 2010). However, there is not enough information available on these enzymes in relation to Penicillium mycotoxicoses. Therefore, it would be interesting to look at the effect of PMs on these enzymes, because changes in cell viability and proliferation are one of the most
common mechanisms of *Penicillium* mycotoxicoses as was previously discussed in this review (Keblys et al., 2004; Oh et al., 2012; Stec et al., 2009).

1.4. **Summary and Conclusion**

Overall, this review provides insight into the potential immunomodulatory effects of PMs through alterations in the viability, proliferation, inflammatory response, cell activation as well as both cytokine and antibody production of immune cells (Keblys et al., 2004; Oh et al., 2012; Xue et al., 2010). PMs differentially affect these parameters of immune response by directly changing cellular activities, such as caspase apoptotic pathways and/or NF-κβ transcriptional pathways (Essid et al., 2012; Ferrante et al., 2006; Kumar et al., 2011). Some researchers have also proposed that PMs affect the cellular activity of immune cells indirectly as a result of alteration in the enzymes regulating epigenetic modifications (Marin-Kuan et al., 2007, 2008). Many *in vitro* studies and also some *in vivo* studies have provided the evidence of their immunomodulatory effects in poultry, rodent and human clinical models (Borrows et al., 2006; Dziarski, 1985; Keblys et al., 2004; Ozsoy et al., 2008; Stoev et al., 2004). Regardless of the fact that ruminants are constantly exposed to PMs from the consumption of ensiled feed (Mansfield et al., 2008), the potential toxic effect of PMs, including immunomodulation, have been disregarded under a general assumption that PMs are rapidly degraded by commensal microbes in the gut (Fuchs et al., 2008; Kiessling et al., 1984). However, there are previously reported cases of various toxicological signs associated with the consumption of PM-contaminated silage in ruminant species (Ciegler, 1977; Häggblom, 1990; Ukai et al., 1954). This suggests that PMs could potentially break through the protection from commensal microbes, potentially leading to immunomodulation, and therefore, possibly leading to secondary diseases, such as Johne’s disease, in ruminant species. In addition
to this, most studies discussed here focused on the risk observed from individual PM exposure, which may not necessarily reflect the true risk of PMs due to their potential interactions among other PMs (Oh et al., 2012; Stoev et al., 2009; Stork et al., 2007; Veselá et al., 1983), because most PMs are often found in combinations of two or more in animal feed (Mansfield et al., 2008). Therefore, in order to gain a greater perspective on how to detect and prevent mycotoxins in the agricultural industry, combinational mycotoxicoses in ruminant species should be considered in future studies.
Table 1.1: The occurrence of *Penicillium* mycotoxins (PMs) in animal feed and food products.

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>location or source, natural condition (nc), artificial condition (ac)</th>
<th>sample material</th>
<th>method used</th>
<th>mean concentrations</th>
<th>Converted concentrations (mg/kg)</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTA</td>
<td>Mexico, conditions created (nc)</td>
<td>corn silage</td>
<td>ELISA</td>
<td>5.1 ppb</td>
<td>0.0051</td>
<td>(Reyes-Velázquez et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>All regions from Portugal (nc)</td>
<td>swine feed</td>
<td>HPLC</td>
<td>11.0 ug/kg</td>
<td>0.011</td>
<td>(Martins et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>laying hen feed</td>
<td></td>
<td>4.0 ug/kg</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>CIT</td>
<td>Calvados, France (nc)</td>
<td>corn silage</td>
<td>HPLC-MS</td>
<td>15.0 ppb</td>
<td>0.015</td>
<td>(Garon et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Normandy, France (nc)</td>
<td>rotten apples</td>
<td>TLC</td>
<td>32.0 ug/kg</td>
<td>0.032</td>
<td>(Richard et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Local store, Portugal (nc)</td>
<td>apple juice</td>
<td>LC-MS</td>
<td>4.74 ng/g</td>
<td>0.005</td>
<td>(Martins et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAT</td>
<td>Across US states (nc)</td>
<td>maize silage</td>
<td>HPLC-MS</td>
<td>0.08 ug/g</td>
<td>0.080</td>
<td>(Mansfield et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Germany (ac)</td>
<td></td>
<td>HPLC; 2D-TLC</td>
<td>15.10 mg/kg</td>
<td>15.100</td>
<td>(Müller and Amend, 1997)</td>
</tr>
<tr>
<td></td>
<td>Local store, Portugal (nc)</td>
<td>rotten apples</td>
<td>TLC</td>
<td>20.45 mg/kg</td>
<td>20.450</td>
<td>(Martins et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Local store, Japan (nc)</td>
<td>apple juice</td>
<td>LC-MS</td>
<td>4.74 mg/kg</td>
<td>0.005</td>
<td>(Kataoka et al., 2009)</td>
</tr>
<tr>
<td>PA</td>
<td>Germany (ac)</td>
<td>maize silage</td>
<td>HPLC; 2D-TLC</td>
<td>3.06 mg/kg</td>
<td>3.060</td>
<td>(Müller and Amend, 1997)</td>
</tr>
<tr>
<td>MPA</td>
<td>Across US states (nc)</td>
<td>maize silage</td>
<td>HPLC-MS</td>
<td>0.16 ug/g</td>
<td>0.160</td>
<td>(Mansfield et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Bavaria, Germany (nc)</td>
<td>grass silage</td>
<td>LC-MS</td>
<td>690 ug/kg</td>
<td>0.690</td>
<td>(Schneweis et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Germany (ac)</td>
<td>maize silage</td>
<td>HPLC; 2D-TLC</td>
<td>2200 ug/kg</td>
<td>2.200</td>
<td>(Müller and Amend, 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.56 mg/kg</td>
<td>3.560</td>
<td></td>
</tr>
<tr>
<td>RQC</td>
<td>Across US states (nc)</td>
<td>maize silage</td>
<td>HPLC-MS</td>
<td>0.38 ug/g</td>
<td>0.380</td>
<td>(Mansfield et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>northern Germany (nc)</td>
<td></td>
<td>HPLC</td>
<td>17.0 mg/kg</td>
<td>17.000</td>
<td>(Auerbach et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Tochigi prefecture, Japan (ac)</td>
<td></td>
<td>TLC</td>
<td>1.66 mg/kg</td>
<td>1.660</td>
<td>(Ohmomo et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Local store, Germany (nc)</td>
<td>Blue cheese</td>
<td>HPLC</td>
<td>1.47 mg/kg</td>
<td>1.470</td>
<td>(Finoli et al., 2001)</td>
</tr>
</tbody>
</table>
Table 1.2: Brief toxicological information of PMs other than immunotoxicity (all references in the context).

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Penicillium spp. Producing mycotoxins</th>
<th>Potential antimicrobial property</th>
<th>Previous reported toxicological signs other than immunotoxicity</th>
<th>LD50s intraperitoneal (ip), intravenous (iv), subcutaneous (sc), oral (po)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTA</td>
<td>P. citrinin, P. verrucosum</td>
<td>No</td>
<td>nephrotoxic (rats, pigs, chicken) hepatotoxic (goat, pigs, chicken) teratogenic (rats)</td>
<td>ip 29.4 mg/kg (mice) po 46.0 mg/kg (mice)</td>
</tr>
<tr>
<td>CIT</td>
<td>P. citrinin, P. verrucosum, P. expansum</td>
<td>Yes</td>
<td>nephrotoxic (pigs) teratogenic (rats) genotoxic (rats)</td>
<td>po 50 mg/kg (mice) ip 35-58 mg/kg (mice) ip 19 mg/kg (rabbits)</td>
</tr>
<tr>
<td>PAT</td>
<td>P. expansum, P. urticae, P. melinii, P. claviforme</td>
<td>Yes</td>
<td>haemorrhagic syndrome (rats, cattles) teratogenicity/mutagenicity (mice) hepatotoxic (rats) destruction of various cell types (rats)</td>
<td>po 17 mg/kg (mice) po 108-118 mg/kg (rats)</td>
</tr>
<tr>
<td>PA</td>
<td>P. puberulum, P. martensii, P. palitans, P. cyclopium</td>
<td>Yes</td>
<td>hepatotoxic (dogs, rats, mice) nephrotoxic (rats, mice) haemorrhagic syndrome (dogs) carcinogenic (rats, mice)</td>
<td>sc 110 mg/kg (rats) iv 250 mg/kg (rats) po 600 mg/kg (rats)</td>
</tr>
<tr>
<td>MPA</td>
<td>P. stoloniferum, P. brevicompactum, P. roqueforti, P. carneurn</td>
<td>Yes</td>
<td>No report of other significant toxic effects than immunotoxicity</td>
<td>po 352 mg/kg (rats) po 1000 mg/kg (mice) po 6000 mg/kg (rabbits)</td>
</tr>
<tr>
<td>RQC</td>
<td>P. roqueforti, P. commune, P. crustosum, P. chrysogenum, P. cyclopium, P. glabrum</td>
<td>Yes</td>
<td>mastitis, abortion, diarrhea (cattles) paralysis, trembling, fatigue (dogs, mice)</td>
<td>ip 20 mg/kg, 169-184 mg/kg (mice)</td>
</tr>
</tbody>
</table>
Table 1.3: Summary of immunomodulatory effect of PMs. (all references in the context; NA-not available)

<table>
<thead>
<tr>
<th></th>
<th>OTA</th>
<th>CIT</th>
<th>PAT</th>
<th>PA</th>
<th>MPA</th>
<th>RQC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target leukocytes</strong></td>
<td>Neutrophils</td>
<td>Macrophages</td>
<td>Monocytes</td>
<td>Macrophages</td>
<td>Macrophages</td>
<td>Macrophages</td>
</tr>
<tr>
<td></td>
<td>Basophils</td>
<td>Lymphocytes</td>
<td>Macrophages</td>
<td>DCs</td>
<td>DCs</td>
<td>Neutrophils</td>
</tr>
<tr>
<td></td>
<td>Monocytes</td>
<td></td>
<td>Lymphocytes</td>
<td>CTLs</td>
<td>NK cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td></td>
<td>Monocytes</td>
<td>Macrophages</td>
<td>activated T-cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NK cells</td>
<td></td>
<td>DCs</td>
<td>Lymphocytes</td>
<td>naive B-cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td></td>
<td>Macrophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Proliferation</strong></td>
<td>↓ (in vitro and in vivo)</td>
<td>↓ (in vitro and in vivo)</td>
<td>↓ (in vitro and in vivo)</td>
<td>↓ (in vitro and in vivo)</td>
<td>↓ (in vitro)</td>
<td></td>
</tr>
<tr>
<td><strong>Cell death</strong></td>
<td>↑ caspase 3/9 apoptotic pathways: activated through TRADD</td>
<td>↑ caspase 3/9 apoptotic pathways</td>
<td>↓ caspase 3/8/9 mediated apoptotic pathways</td>
<td>↑ necrosis due to oxidative stress at concentration higher than 100 μM (in vitro)</td>
<td>Severely affect the cells (activated T-cells &amp; naive B-cells) utilizing type II IMPDH for GTP synthesis</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>↑ necrosis due to oxidative stress</td>
<td>↑ necrosis mediated by oxidative stress due to GSH-based antioxidants</td>
<td>↑ necrosis due to oxidative stress</td>
<td>↑ necrosis due to oxidative stress at concentration higher than 100 μM (in vitro)</td>
<td>May act as an irritant</td>
<td>↑ MIP-2, TNF-α in BALF</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td>↑ NF-κβ and MAPK Pathways</td>
<td>↑ NF-κβ and MAPK Pathways</td>
<td>↓ IL-1β, IL-6, TNF-α, IL-10, ROS production</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>↑ COX-2, iNOS, hsp72, TNF-α</td>
<td>↑ COX-2, iNOS, hsp72, TNF-α, NO</td>
<td>↑ MAPK pathways</td>
<td>↑ ROS production</td>
<td>↑ trafficking of DCs to inflammatory sites by ↑ CCR-1, ↑ IL-10, TGF-β</td>
<td></td>
</tr>
<tr>
<td><strong>Cell-mediated immune response</strong></td>
<td>↓ IFN-α, IFN-β, IFN-γ</td>
<td>↓ IFN-γ</td>
<td>↓ IFN-γ, IL-12, IL-2</td>
<td>NA</td>
<td>↓ activation of NK cells, DCs, T-cells</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>↓ phagocytosis, antitumor activity of NK cells, basophil hypersensitivity, DTH response</td>
<td>↓ infectivity of intracellular pathogens (T. gondii)</td>
<td>↓ phagocytosis and microbicidal activity to S. cerevisiae</td>
<td>↑ T-reg cell number</td>
<td>↑ intracellular infection</td>
<td></td>
</tr>
<tr>
<td><strong>Antibody-mediated immune response</strong></td>
<td>↓ IgM and IgG (due to ↓ cell number)</td>
<td>↓ IL-4 (less sensitive compared to IFN-γ)</td>
<td>↓ IL-4 (less sensitive compared to IFN-γ)</td>
<td>NA</td>
<td>↓ activation B-cells</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ antibody response to antigens (sRBC, ovalbumin)</td>
<td>↓ IgM, IgA, IgG</td>
<td>↑ IgG1/IgG2 ratio</td>
<td>↓ IgM, IgA, IgG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ IgM, IgA, IgG</td>
<td></td>
<td>↑ IL-5</td>
<td>No effect of PCs</td>
</tr>
</tbody>
</table>
B. In the absence of PMs

A. In the presence of PMs

Figure 1.1: Simple schematic diagram of how the antimicrobial properties of PMs could disrupt the homeostasis of intestinal immune system. A. In the absence of PMs versus B. In the presence of PMs.
Figure 1.2: The Schematic diagram of how PMs alter inflammatory response of macrophages by altering HDAC-3 (Adapted from Rahman et al., 2004). Green arrow represents ‘stimulation’, and red indicates ‘inhibition.’
Chapter 2: Experimental Rationale, Hypothesis, and Objectives

2.1. Experimental Rationale

Penicillium molds are one of common fungal species that contaminate a wide variety of cereal and livestock feed crops worldwide (Almeida et al., 2000; Creppy, 2002; Duong, 1996). Many of these mold species can produce biologically active secondary metabolites, commonly known as 'mycotoxins.' Poor storage conditions for most grains and food products such as moderate temperature with high humidity, often provide favourable conditions for the production of Penicillium mycotoxins (PMs) (Dalcero et al., 1998; Mansfield et al., 2008), adversely affect the safety of both human and animal feeds (Miraglia et al., 2009). Due to their dramatic increase during ensilaging of animal feed, PMs are also refereed as ‘silage mycotoxins.’

There are several PMs found in stored feed or food, such as citrinin (CIT), ochratoxin (OTA), patulin (PAT), mycophilic acid (MPA) and penicillic acid (PA). Studies have found that these PMs have a variety of toxicological effect in animals (Dickens and Jones, 1965; Ferrante et al., 2008; Sansing et al., 1976). For example, some mycotoxins, such as OTA, CIT and PA, are nephrotoxic (Braunberg et al., 1992; Sansing et al., 1976) and hepatotoxic (Dickens and Jones, 1965) in monogastric species, while PAT is a potent hepatotoxicant in both monogastric and ruminant species (Hayes et al., 1979; Sommer et al., 1974; Ukai et al., 1954). Some studies have also reported that these mycotoxins are potential immunomodulators, which could predispose animals to secondary diseases (Al-Anati and Petzinger, 2006b; Ferrante et al., 2008; Herzog-Soares and Freire, 2004).

In terms of their immunomodulatory activity, PMs range from being inducers of immune cell apoptosis or necrosis, and altering activities of local or systemic immune responses. Most of these PMs have been shown to reduce the proliferation and viability of immune cells under similar or
different mechanisms of action. CIT, OTA and PAT for example, induced cellular oxidative stress and ROS production, which can trigger the apoptosis or necrosis of leukocytes, and therefore, decreasing the cell number (Essid et al., 2012; Kumar et al., 2011; Wu et al., 2008). PA, on the other hand, has been reported to prevent apoptosis, but also decreased cell viability by necrosis of lymphoma cell line (Bando et al., 2003). In addition to this, these PMs have also been shown to inhibit the proliferation of bovine as well as porcine lymphocytes (Keblys et al., 2004; Stec et al., 2008).

Other studies have also reported that these PMs at their sub-lethal levels can modulate the function of immune cells. Ferrante et al. (2006, 2008) for example, reported that OTA was a potential inducer of inflammatory molecules, such as cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and heat shock protein 72 (hsp72), in murine macrophages. In contrast to OTA, MPA is a well-known immunosuppressive drug that has been used for treating inflammatory disorders such as arteriosclerosis and rheumatoid arthritis (Cohn et al., 1999; Mohacsi et al., 1997), as well as preventing organ rejection after transplantation (Weber et al., 1998). In addition to this, OTA when combined with either CIT or PA was previously shown to synergistically enhance the toxicity of these mycotoxins to liver and kidney, as well as macrophage (Sansing et al., 1976; Veselá et al., 1983; Oh et al. 2012).

There are hundreds of mycotoxins, produced from many different mold species, and only a few of them, specifically those from either *Fusarium* or *Aspergillus* species, have been thoroughly studied and are known to pose a human and animal health risk. Potential risk of mycotoxicoses caused by PMs, on the other hand, has been disregarded regardless of the high prevalence of PMs in stored fruits and crops for human and animal consumption, and their potential to impact health (Häggblom et al., 1990; Kulik and Holaday, 1966; Pitt, 1987). Therefore, it is important that we
understand the risk posed by exposure to these bioactive compounds; to date relatively little is known about the risk of exposure to PMs. Regardless of their abundance in animal feed and their potential to affect the immune system, the agricultural and economic importance of PMs are often under-estimated because it is generally assumed that PMs are readily metabolized by gut microbiota and hepatic enzymes (Abrunhosa et al., 2010; Fuchs et al., 2008). However, considering that most of these PMs have anti-microbial properties (Kavanagh, 1947) and are found to be hepatotoxic to animals (Dickens and Jones, 1965; Hayes et al., 1979; Sansing et al., 1976), it is possible that detoxification of the PMs is attenuated because of these properties. Furthermore, PMs may also enter the circulation system and target tissues without passing through the digestive tract. For example, it has been reported that mycotoxins can enter circulation during inhalation (Halstensen et al., 2004; Skaug et al., 2001) and dermal exposure (Panda et al., 1997). Since entry via these routes bypasses initial detoxification provided by gut microbiota and the liver, exposure via inhalation and dermal contact could be a serious health risk for farmers as well as their livestock (EFSA, 2012; Reijula and Tuomi, 2003; Smith et al., 1995).

In this thesis, we have used a bovine macrophage cell line (BoMacs; provided by Stabel and Stabel, 1995) as an in vitro cell culture model to test the immunomodulatory effect of PMs on macrophages. Macrophages have been widely used as an in vitro model to assess the potential effect of mycotoxins on immune responses (Ferrante et al., 2008; Islam et al., 2012; Sorenson et al., 1986; Wigginton et al., 2001) because they are one of the key immune mediators that bridge between innate and adaptive immune systems (Mosser, 2003). Disruption of the normal function of macrophages can alter the activities of other immune cells and mediators, potentially leading to abnormal immune response and therefore, progression of secondary diseases, such as rumatoid arthritis, tumor development or other secondary infections (Ma and Pope, 2005; Mosser, 2003;
Pollard, 2004). Therefore, looking at the immunomodulatory effect of PMs on BoMacs, which are anatomically relevant because they are derived from peritoneal macrophages (Stabel and Stabel, 1995), would provide better understanding of the potential risk of PM exposure to macrophages functions, and therefore their potential impact on intestinal immune health in bovine species.

2.2. Experimental Objectives

1) To determine the toxic ranges of individual PMs using BoMac proliferation and viability and the potential interaction of binary mixtures of two. Published in *The Open Mycology Journal* 2012; 6(1): 11-16.

2) To determine if *in vitro* exposure to individual and combined PMs alters the expression of genes involved in epigenetic regulation, including DNA methyltransferases (DNMT1, 2, 3a, and 3b), histone demethylases (JMJD3 and UTX) as well as a histone acetylase (Bmi-1) and histone deacetylases (HDACs) in BoMacs. Published in *Mycotoxin Research* 2013 Nov; 29(4): 235-43. With contribution of two undergraduate research project students, Caroline G. Balch and Rachael L. Cliff.

3) To investigate the potential effect of PMs on BoMacs function including cytokine gene expression, ROS production and phagocytosis of *Mycobacterium avium* subspecies *paratuberculosis* (MAP).

4) To assess the efficacy of Mycosorb A+ (MA+) for preventing PMs toxicity using BoMac proliferation as a common endpoint.

5) Re-examination of the interactions of the binary mixtures of mycotoxins by applying an independent action (IA) and a concentration addition (CA) model.
2.3. Experimental Hypotheses

1) PMs alter the proliferation and cell viability of BoMacs in a dose-dependent manner, and certain combinations of these mycotoxins interact to further inhibit the cell proliferation.

2) The process of PM-induced changes in gene expression is attributed, in part, to changes in the expression of genes that encode enzymes involved in epigenetic modifications such as DNA methylation as well as histone demethylation, acetylation and deacetylation.

3) PMs differentially alter the gene expression of BoMacs cytokines, and this in turn, is reflected by changes in the activities of the macrophages, including ROS production and phagocytosis of MAP.

4) Different factors such as pH, incubation time and percent concentration of MA+ would differentially affect the efficacy of MA+ for preventing PM toxicity.

5) The IA or CA model can be applied to predict the interactions of certain binary mixtures of PMs, including synergism or antagonism, and these models may provide better estimation of the true risks associated with PM mixture toxicity.
Chapter 3: Immunotoxicity of *Penicillium* Mycotoxins on Viability and Proliferation of Bovine Macrophage Cell Line (BoMacs)

Published: Oh S-Y, Boermans HJ, Swamy HVLN, Sharma BS and Karrow NA. The Open Mycology Journal 2012; 6(1): 11-16. doi: 10.2174/1874437001206010011 (Figures, Tables, References formatted to fit for the thesis)

### 3.1. Abstract

*Penicillium* mycotoxins are natural contaminants found in grains, crops, fruits, and fermented products, especially during post-harvest as well as storage periods. Contamination by individual and combinations of these toxins is likely to compromise food quality and safety. In this study, the potential immunotoxicity of citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA) and penicillic acid (PA) was evaluated using a bovine macrophage cell line (BoMacs) by assessing their potential cytotoxicity and then their effects on cell proliferation. The BoMacs were exposed to a range of mycotoxin concentrations, and then to different mycotoxin combinations for 48 hrs. Some cytotoxicity was evident at concentrations greater than 2.4 µM for PAT, and 160 µM for PA, however, at the IC50 (concentration that inhibits 50% cell proliferation), no cytotoxicity was observed for either of these mycotoxins. The mycotoxin IC50s from most potent to least potent were 0.56 µM (PAT), 12.88 µM (OTA), 29.85 µM (PA), and 91.20 µM (CIT). Concentrations of MPA greater than 80 µM did not inhibit cell proliferation enough to calculate an IC50. Significant higher inhibition of cell proliferation was observed from the combinations of CIT+OTA, OTA+PAT, and OTA+PA compared to the effects of individual mycotoxins suggesting additive and in some cases synergistic activity between these paired mycotoxins.
3.2. Introduction

Mycotoxins are metabolites of filamentous fungi that naturally contaminate a wide variety of crops, and therefore cereals and livestock feeds, worldwide (Placinta et al., 1999; Tanaka et al., 1988). The Food and Agriculture Organization of the United Nations (FAO) estimated that approximately 25% of the food and food stuffs in the world are compromised by mycotoxin contamination (Park et al., 2009). Economic losses from mycotoxin contamination occur due to reduced crop yield and value, and reduced livestock productivity and animal health from the consumption of contaminated feed (Schmale III and Munkvold, 2009). Environmental conditions associated with high humidity and temperature favor fungal contamination of feed stuffs, and this can significantly affect the safety of both human and animal feed (Miraglia et al., 2009). The effects of individual mycotoxins, such as carcinogenicity, teratogenicity, genotoxicity, nephrotoxicity as well as immunotoxicity have been studied, but their combinational effects have not been assessed (Ciegler et al., 1977; Keblys et al., 2004; Liu et al., 2003). One of the difficulties associated with characterizing the toxicity of mycotoxins is that contaminated feedstuffs may contain various combinations of mycotoxins produced by different fungal species (Mansfield et al., 2008). For example, *Penicillium expansum* produces citrinin (CIT), ochratoxin A (OTA), and patulin (PAT), while *Aspergillus ochraceus* produces OTA and PA (Andersen et al., 2004; Martins et al., 2002; Sansing et al., 1976). Additionally, *Fusarium* mycotoxins are typically more abundant in corn silage immediately post-harvest, whereas *Penicillium* mycotoxin contamination increases during storage (Haladi, personal communication).

Among many fungal species, the *Penicillium*, *Aspergillus*, and *Fusarium* fungi are the most predominant species found in livestock feed such as corn silage (Amiri and Bompeix, 2005; Dalcero et al., 1997; Pereyra et al., 2008; Pozzi et al., 1995). Many studies have characterized the
impact of oral exposure to *Fusarium* and *Aspergillus* mycotoxins on livestock species and examples of genotoxicity and immunotoxicity have been reported (Amiri and Bompeix, 2005; Visconti et al., 1991). However, little is known about the impact of exposure to *Penicillium* mycotoxins, despite their potential to modulate immune function (Keblys et al., 2004; Richetti et al., 2003; Stec et al., 2008). It is assumed that mycotoxins in general are detoxified by rumen microflora (Kiessling et al., 1984), since commensal microbes within ruminants have capacity to neutralize mycotoxins produced from *Fusarium* or *Aspergillus* mold before they enter the systemic circulation via the hepatic portal vein (Swanson et al., 1987). Many *Penicillium* mycotoxins on the other hand, are known to have anti-microbial properties, which could disrupt the normal function of these microbes, including their detoxification of *Penicillium* mycotoxins (Bennett and Klich, 2003; Dzidic et al., 2010). Therefore, *Penicillium* mycotoxin exposure may be especially relevant to the health of ruminant species, especially since they are frequently found in contaminated silage.

In this study, we evaluated the cytotoxicity and combined effect of exposure to CIT, OTA, PAT, mycophenolic acid (MPA), and penicillic acid (PA) on the proliferation of a bovine macrophage cell line (BoMacs). We hypothesized that *Penicillium* mycotoxins will alter the proliferation of BoMacs in a dose-dependent manner, and that certain combinations of these mycotoxins will additively and/or synergistically inhibit the cell proliferation.

3.3. Material and methods

3.3.1. Cell Preparation and Mycotoxin Exposure

The BoMacs (provided by Stabel and Stabel, 1995) were cultured in RMPI 1640 supplemented with 2.0 mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS), 100 unit/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and 25 mM HEPES buffer (Invitrogen, ON, Canada). After reaching confluence, the BoMacs were dislodged
with 0.05% trypsin-EDTA (Invitrogen, ON, Canada), seeded into 96-well flat bottom plates (10,000 cells per well), and incubated at 37°C with 5% CO₂ for 1 h. The BoMacs were then exposed to a range of concentrations of CIT, OTA, PAT, MPA, and PA (Sigma, ON, Canada, Table 3.1) dissolved in dimethylsulfoxide (DMSO) for 47 h at 37°C with 5% CO₂. The ranges of exposure concentration were selected based on cited IC50s determined for bovine and porcine lymphocytes (Keblys et al., 2004; Stec et al., 2008), and several preliminary trials using BoMacs that were used to optimize the toxic range of concentrations for each mycotoxin.

3.3.2. Assessing Cytotoxicity of CIT, OTA, PAT, MPA, and PA to BoMacs

After 47 h of mycotoxin exposure, another batch of BoMacs was seeded into 96-well plates (50,000, 37,500, 25,000, 12,500, 0 cells per well) and incubated for 1 h at 37°C with 5% CO₂; these cells were used as viable standards for the cytotoxicity assay. A parallel standard for dead cells was also prepared by incubating BoMacs in 70% methanol at room temperature for 1 h; these cells were stained with 0.1% trypan blue and viewed under a light microscope to confirm cell death. The standard for dead cells was prepared by seeding the dead cells into the 96-well plates already containing the standard for live cells such that the live cell proportion was equivalent to 100, 75, 50, 25, and 0%. At 48 h post mycotoxin exposure, all plates including the standards were washed with PBS, and cytotoxicity was assessed using a commercially available cytotoxicity kit (Invitrogen, ON, Canada). The number of live cells was estimated using a 1420 Victor-2 Multilabel Counter (Beckman Coulter, Inc. California) by measuring the fluorescence of calcein AM (excitation 494/emission 517 nm), once it was hydrolyzed by intracellular esterases, and the number of dead cells was estimated by measuring the fluorescence of ethidium homodimer-1 (excitation 528/emission 617 nm) bound to nucleic acids. When possible, the LC50 (concentration
that kills 50% of cells) was calculated for each mycotoxin using the Graphpad Prism software (La Jolla, California, U.S.A.).

3.3.3. Assessing the Inhibitory Effect of CIT, OTA, PAT, MPA, and PA on BoMac Proliferation

The BoMacs were exposed to the mycotoxins as described above, and after 47 h of exposure, a standard for the proliferation assay was prepared by seeding another batch of BoMacs (50,000, 25,000, 12,500, 6,250, 3,125, 1,563, 782, 0 per well) into the 96-well plates for 1 h of incubation at 37°C with 5% CO₂. The media was then removed from all plates by blotting, and the plates were frozen for 24 h at -80°C prior to the proliferation assay. The number of cells was estimated using a commercially available proliferation kit (Invitrogen, ON, Canada) that uses CyQUANT® GR dye (excitation 494/emission 517 nm) to label nucleic acids. The fluorescence intensity was measured with a 1420 Victor-2 Multilabel Counter. The IC50 (concentration that inhibits 50% cell proliferation) for each mycotoxin was calculated using the Graphpad Prism software.

3.3.4. Assessing the Combined Effects of Penicillium Mycotoxin on BoMac proliferation

The IC25 for each mycotoxin was calculated using the Graphpad Prism software and is shown in the toxicity curves in Figure 3.1. The BoMacs were then subjected to exposure by various combinations of CIT, OTA, PAT, MPA, and PA at their respective IC25s and to the individual mycotoxins (Table 3.2); cell proliferation was then assessed as described above.

3.3.5. Data Analysis

All data are presented as percent cytotoxicity and percent cell proliferation, based on the combined results of three independent experiments. For the dose-response data, an ANOVA followed by Dunnett’s test was used to test for statistical significance between the solvent controls
and treatments (b), and a T-test was used to make comparisons between the control and solvent control (a). For the combined mycotoxins, an ANOVA followed by Tukey’s test for comparisons was used to compare treatment groups (c) (Figure 3.2). The concentration data were log transformed for the analysis, and significance was determined at a p-value ≤ 0.05 using the Graphpad Prism software.

3.4. Result and Discussion

3.4.1. Cytotoxicity and Inhibition of the Cell Proliferation due to Individual Mycotoxin Exposure

Cytotoxicity was evident at concentrations greater than 2.4 µM for PAT, and 160 µM for PA; the LC50s for PAT and PA were estimated to be 4.46 µM and 175.79 µM, respectively (Table 3.2). The cytotoxicity of PAT and PA may be due to their genotoxic effect on BoMacs. In support of this, Liu et al. (2003) concluded PAT as a potent clastogen with the ability to cause oxidative damage to DNA in hamster ovary cells and human peripheral blood lymphocytes, while CIT did not exhibit any significant evidence of such genotoxicity (Liu et al., 2003). In the present study, CIT, OTA, and MPA did not induce any cytotoxicity within the concentration ranges tested (Figure 3.1A). It is possible that higher concentrations of these mycotoxins would have induced sufficient cytotoxicity to estimate their respective LC50s, however, the concentration of DMSO at these higher concentrations would have also inhibit the proliferation of BoMacs, biasing the results of the proliferation assay (Data not shown).

In the context of cell proliferation, the mycotoxin IC50s calculated in this study occurred at concentrations where no overt cytotoxicity occurred, and their order of potency from highest to lowest was: 0.56 µM (PAT) > 12.88 µM (OTA) > 29.85 µM (PA) > 91.20 µM (CIT) (Table 3.2).
When these IC50s were compared to IC50s determined from other studies, it appears that BoMacs are less sensitive to CIT, OTA, and PA, but are more sensitive than porcine and bovine lymphocytes to PAT (Table 3.3). The exposure concentrations of some of the mycotoxins investigated and IC50s determined in this study, such as OTA, were higher than circulating concentrations reported from in vivo studies (Blank et al., 2003; Höhler et al., 1999). However, there is the potential for their bioaccumulation in certain target tissues, such as kidney, intestine, and cutaenous fat (Galtier et al., 1981; Prelusky et al., 1986), as well as their synergistic and additive interaction among other mycotoxins within these tissues (Sansing et al., 1976).

Based on the observations of this study, MPA was found to have little effect on BoMacs. Although MPA concentrations of 32 µM and 320 µM did inhibit cell proliferation, the response was not significantly different between these concentrations, and was not substantial enough to estimate an IC50 (Figure 3.1B). The inhibitory mechanism of MPA on cell proliferation has been well studied. Mycophenolic acid and its commercial analogue, mycophenolate mofetil (MMF), block the de-novo purine biosynthesis pathway, which is required for lymphocyte proliferation, by reducing cellular NF-kB levels (Hauser et al., 1997). While the proliferation of B- and T-lymphocytes relies heavily on the de novo pathway, BoMacs may equally utilize both de-novo and salvage pathways for their proliferation (Duncan and Wilkes, 2005). Furthermore, a more gradual reduction in cell proliferation was observed at the lower MPA concentrations when compared to the other mycotoxins (Figure 3.1B). These distinct characteristics may suggest why MPA and MMF have been successfully used for treatment of allogenic transplantation as well as other immune-mediated diseases (Chaudhry et al., 2001; Pérez-Simón et al., 2008).
3.4.2. Combined Effect of Penicillium Mycotoxins on the Proliferation of BoMacs

When the ten different combinations of mycotoxins were evaluated in the present study, three combinations significantly inhibited the proliferation of BoMacs when compared to the individual mycotoxins. These combinations included; CIT+OTA, OTA+PAT, and OTA+PA, (Figure 3.2). Combinations of CIT, OTA, and PA were of interest because these mycotoxins can be produced by Penicillium viridicatum, whereas Aspergillus ochraceus produces OTA and PA (Bacon et al., 1973; Sansing et al., 1976). Penicillium expansum, which is commonly found in rotten apples, is also capable of producing CIT, PAT, and OTA (Andersen et al., 2004; Martins et al., 2002). The combination of CIT+OTA was previously demonstrated to increase chicken embryonic toxicity compared to CIT or OTA, alone (Veselá et al., 1983). Additionally, Sansing et al. (1976) reported that CIT+OTA and OTA+PA inhibited orotic acid incorporation into liver and kidney tissues, while this inhibitory effect was not observed from any of the individual mycotoxins (Sansing et al., 1976). Furthermore, the synergistic and additive interaction between CIT and OTA as well as OTA and PA on nephrotoxicity, hepatotoxicity and teratogenicity has been well addressed in other studies (Shepherd et al., 1981; Speijers and Speijers, 2004). In the present study, the proliferation of BoMacs was shown to be inhibited in an additive manner with the combinations of CIT+OTA and OTA+PAT, while the synergistic activity was observed from the combination of OTA+PA (Figure 3.2).
3.5. Summary and Conclusion

The inhibitory effect of the following *Penicillium* mycotoxins, CIT, OTA, PAT, MPA, and PA, as well as their combinations, on the cytotoxicity and proliferation of BoMacs was evaluated in this study. All mycotoxins except MPA inhibited cell proliferation by at least 50% with the evidence of cytotoxicity from PAT and PA for the concentration ranges used in this study. Most of these toxic concentrations determined in the study are higher than circulating concentrations from *in vivo* studies, yet the continuous consumption of feed contaminated with these toxins may lead to their accumulation in host tissues potentially increasing their bioavailability to the host immune cells. Of greater concern, however, was the additive or synergistic effects of various combinations of mycotoxins including; CIT+OTA, OTA+PAT, and OTA+PA. Since these mycotoxins can be naturally found in these combinations, their effects on the immune system warrant further investigation.
Table 3.1: Mycotoxin concentrations used for BoMac exposure study (µM)

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>Concentrations (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIT</td>
<td>2.5, 5, 10, 20, 30, 40, 60, 80, 160, 320</td>
</tr>
<tr>
<td>OTA</td>
<td>0.30, 0.60, 1.2, 2.4, 4.8, 7.0, 9.6, 14, 19.2, 38.4</td>
</tr>
<tr>
<td>PAT</td>
<td>0.0038, 0.0075, 0.015, 0.038, 0.075, 0.15, 0.2, 0.3, 0.4, 0.5, 0.6, 1.2, 2.4, 4.8</td>
</tr>
<tr>
<td>MPA</td>
<td>0.05, 0.10, 0.2, 0.4, 0.6, 0.8, 1.6, 3.2, 32, 160, 320</td>
</tr>
<tr>
<td>PA</td>
<td>2.5, 4, 5, 10, 15, 20, 40, 80, 160, 320</td>
</tr>
</tbody>
</table>
Table 3.2. The LC50, IC50, and IC25 of various *Penicillium* mycotoxins (µM). LC50s estimated from Figure 3.1A; IC 50s and IC25s estimated from Figure 3.2B. *R-square: the fitness of curve with the data points from Figure 3.2B.

<table>
<thead>
<tr>
<th></th>
<th>CIT</th>
<th>OTA</th>
<th>PAT</th>
<th>MPA</th>
<th>PA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LC50</strong></td>
<td>N/A</td>
<td>N/A</td>
<td>4.46</td>
<td>N.A</td>
<td>175.79</td>
</tr>
<tr>
<td><strong>IC50</strong></td>
<td>91.20</td>
<td>12.88</td>
<td>0.56</td>
<td>N/A</td>
<td>29.85</td>
</tr>
<tr>
<td><strong>IC25</strong></td>
<td>52.72</td>
<td>8.91</td>
<td>0.32</td>
<td>0.50</td>
<td>13.90</td>
</tr>
<tr>
<td><strong>R-Square</strong></td>
<td>0.945</td>
<td>0.981</td>
<td>0.841</td>
<td>0.871</td>
<td>0.987</td>
</tr>
</tbody>
</table>
Table 3.3: Comparison of Mycotoxin IC50s for cell proliferation of porcine, bovine lymphocytes, and BoMacs (µM).

<table>
<thead>
<tr>
<th></th>
<th><strong>Porcine Lymphocyte</strong></th>
<th><strong>Bovine Lymphocyte</strong></th>
<th><strong>BoMacs</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Keblys et al., 2004)</td>
<td>(Stec et al., 2008)</td>
<td></td>
</tr>
<tr>
<td>CIT</td>
<td>38.00</td>
<td>44.27</td>
<td>91.20</td>
</tr>
<tr>
<td>OTA</td>
<td>1.30</td>
<td>4.41</td>
<td>12.88</td>
</tr>
<tr>
<td>PAT</td>
<td>1.20</td>
<td>3.63</td>
<td>0.56</td>
</tr>
<tr>
<td>PA</td>
<td>18.00</td>
<td>N/A</td>
<td>29.85</td>
</tr>
</tbody>
</table>
Figure 3.1: A. Cytotoxicity and B. Proliferation of BoMacs following 48 h of Mycotoxin Exposure. CIT(□), OTA(△), PAT(▽), and MPA(◇), PA(○). LC50s (the concentration that kills 50% of cells) are indicated in A; IC25s (The concentrations that inhibits 25% cell proliferation) and IC50s (The concentrations that inhibits 50% cell proliferation) are indicated in B with dotted lines.
Figure 3.2: The combined Effects of Mycotoxins on the Proliferation of BoMacs. Solvent control 1 is only for the combinations of CIT+OTA. Solvent control 2 is for all other combinations and individual mycotoxins. (a) control vs solvent controls; (b) the solvent controls vs individual treatments; (c) combined vs individual treatments.
Chapter 4: Exposure to *Penicillium* Mycotoxins Alters Gene Expression of Enzymes Involved in the Epigenetic Regulation of Bovine Macrophages (BoMacs)


4.1. Abstract

In this study, the modulation of key enzymes involved in epigenetic regulation was assessed in immortalized bovine macrophages (BoMacs) following *in vitro* exposure to the following *Penicillium* mycotoxins (PMs): citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA), penicillic acid (PA), or a combination of one of the above with OTA at the concentration that inhibits BoMac proliferation by 25% (IC25). Real-time PCR analysis of the genes coding DNA methyltransferases (DNMTs), histone demethylases (JMJD-3 and UTX), as well as the class-1 histone deacetylases (HDAC-1, -2, and -3) and histone acetylase (Bmi-1) was assessed following 6 h of mycotoxin exposure. A change in the expression of JMJD-3 as well as HDAC-3, MPA (p=0.1) and PA (p=0.08), by at least one of the treatments was observed at their respective IC25. The expression of JMJD-3 was significantly induced by PA, but synergistically suppressed by CIT+OTA. The combination of CIT+OTA also synergistically suppressed the expression of DNMT-3a and DNMT-3b. The combination of PAT+OTA reduced DNMT-3a expression, while PA+OTA reduced DNMT-3b expression. Lastly, MPA and PA slightly reduced HDAC-3 expression, while OTA in combination with CIT, PAT, MPA or PA synergistically suppressed HDAC-3 expression. The results of this study demonstrate that PM exposure,
specifically OTA and other mycotoxin combinations, can alter the expression of BoMac enzymes that are involved in epigenetic regulation. These findings suggest a potential novel regulatory mechanism by which mycotoxins can modulate macrophage function.

4.2. Introduction

Mycotoxins are secondary metabolites produced by microfungi that are capable of causing toxic effects in animals (Bennett and Klich, 2003). In the context of livestock production, subacute or chronic mycotoxicosis decreases food efficiency, fertility or overall health status (Diekman and Green, 1992), which could potentially lead to substantial economic losses (CAST, 2003).

The following *Penicillium* mycotoxins (PMs): citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA), and penicillic acids (PA) frequently contaminate crops, grains, feeds and fruits of Northern Europe and North and South America as well as East Asia (FAO, 2003; Flajs and Peraica, 2009; Kang et al., 2007; Mansfield et al., 2008; Tangni and Pussemier, 2006; Yue et al., 2011). For example, *P. verrucosum* is a major producer of CIT and OTA in stored grains (Frisvad et al., 2005), while CIT and PAT are produced by *P. expansum*, which can be found in fruits and cereal grains (Ciegler, 1977; Martins et al., 2002). Some of *Aspergillus* species are also capable of producing these mycotoxins. However, Frisvad et al. 2004 suggested that the combined effect of *P. verrucosum* and *P. viridicata* is a more common source of co-contamination of OTA and PA in cereal grains rather than *Aspergillus ochraceus* (Frisvad et al., 2004, 2005). Furthermore, the concentration of these mycotoxins from *Penicillium spp.* tends to dramatically increase during the post harvest, especially during the storage periods (Mansfield et al., 2008; Sweeney and Dobson, 1998).
Some of these PMs, including MPA, PAT, and PA have immunomodulatory properties in addition to their antimicrobial properties (Bennett and Klich, 2003; Dzidic et al., 2010). For example, *in vitro* exposure of rat alveolar macrophages to PA or PAT resulted in numerous toxic effects including inhibited protein and RNA synthesis, which is likely to interfere with macrophage function (Sorenson et al., 1986). MPA, on the other hand, selectively suppresses cell proliferation and the differentiation of immune cells, such as T- and B-lymphocytes (Dzidic et al., 2006). OTA modulates the production of specific cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) (Dhuley, 1997), and also induces immune cell death (Al-Anati and Petzinger, 2006b). CIT is often found in combination with OTA and acts synergistically with OTA to inhibit RNA synthesis of specific proteins that alter cellular function (Sansing et al., 1976).

Each of the mycotoxins discussed herein have shown evidence of causing cellular disruption by altering of RNA and protein synthesis. We hypothesize that the process of mycotoxin-induced changes in gene expression is attributed, in part, to changes in the expression of genes that encode enzymes involved in epigenetic modifications such as DNA methylation as well as histone demethylation, acetylation and deacetylation.

Epigenetics refers to heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence. This change is accomplished by modification of chromatin either by DNA methylation or covalent modification of histones (e.g. acetylation, deacetylation, or methylation). Changes in epigenetic patterns can occur throughout an individual’s life in response to both internal and external stimuli (Delcuve et al., 2009).

The process of DNA methylation is catalyzed by a family of enzymes called DNA methyltransferases (DNMTs): DNMT1, DNMT2, DMNT3a, and DNMT3b (Miranda and Jones, 2007). Changes in the methylation pattern can also occur on histone proteins that help to package
the DNA by demethylases, such as Jumonji domain-containing protein 3 (JMJD3) and tetratricopeptide-repeat X (UTX). Histone deacetylases (HDACs), such as HDAC-1, HDAC-2, and HDAC-3, are enzymes that remove acetyl groups (O=C-CH₃) from N-terminal of internal lysine on histone protein 3 and/or 4 (Kuo and Allis, 1998). Polycomb ring finger oncogene (bmi-1) is part of polycomb repressor complex (PRC1) that has ubiquitin E3 ligase as well as histone acetylase activities (Sparmann and van Lohuizen, 2006). The abnormal activity of these enzymes is closely linked to various diseases, such as neurological diseases (Fischer et al., 2010), cancer (Qin et al., 2009), and immune disorders (Royce and Karagiannis, 2012).

Many macrophage cell functions are under the control of epigenetic modifications and this regulation is crucial for a successful cell activity and inflammatory response (Bowdridge and Gause, 2010). The characteristic marker genes of anti-inflammatory M2 macrophages for example, are epigenetically regulated (Ishii et al., 2009). It is possible that mycotoxins induce changes in gene expression by influencing epigenetic mechanisms as opposed to acting directly on target gene transcription. For example, Marin-Kuan et al. (2008) hypothesized that the carcinogenicity of OTA is likely due to the disruption of epigenetic mechanisms as opposed to a direct action of OTA on the DNA. Several studies have shown that PM exposure disrupts normal activities of immune cells, such as proliferation and inflammation (Dhuley, 1997; Dzidic et al., 2010; Oh et al., 2012; Sorenson et al., 1986), and this may be the result of changes in epigenetic enzyme expression. The present study was designed to investigate the effects of PM exposure on the gene expression of enzymes responsible for epigenetic modifications using bovine macrophage cells (BoMac). Specifically, the objective of the present study was to determine if in vitro exposure to individual and combined mycotoxins alters the expression of genes coding DNA methyltransferases (DNMT1, 2, 3a, and 3b), histone demethylases (JMJD3 and UTX) as well as a histone acetylase
(Bmi-1) and histone deacetylases (HDACs) in BoMacs. Changes in expression of these genes may indirectly reflect global changes in DNA methylation and histone methylation.

4.3. Material and methods

4.3.1. Mycotoxins

The following PMs were obtained from Sigma (Ontario, Canada) and dissolved in dimethyl sulfoxide (DMSO) to a stock concentrations: citrinin (CIT; 20.0 mM), ochratoxin A (OTA; 2.5 mM), patulin (PAT; 32.4 mM), mycophenolic acid (MPA; 156.1 mM), and penicillic acid (PA; 58.8 mM).

4.3.2. BoMac culture

The BoMacs (provided by Stabel and Stabel, 1995) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 2.0 mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS), 100 unit/ml of penicillin, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B, and 25 mM HEPES buffer. Cells were incubated at 37°C with 5% CO₂ in 75 cm² flask prior to use.

4.3.3. Cell preparation and mycotoxin exposure

BoMacs (1 x 10⁶) were seeded into six-well flat-bottom plates (Corning Inc., U.S.A), centrifuged for 2 min and incubated at 37 °C with 5% CO₂ for 60 min. After incubation, the cells were stimulated with 1 μg/ul of Escherichia coli lipopolysaccharide (LPS) (Escherichia coli O111:B4, Sigma, Oakville, Canada) and exposed immediately to the PMs at their respective IC25, the concentration that inhibits cell proliferation by 25%. The IC25s were determined from a previous study with BoMacs and cell viability was not adversely affected at these concentrations (Oh et al., 2012). 52.72 μM for CIT, 8.91 μM for OTA, 0.32 μM for PAT, 0.50 μM for MPA, and
13.90 μM for PA. Following combinations of mycotoxins, CIT+OTA, PAT+OTA, MPA+OTA and PA+OTA, were also selected as these combinations were previously shown to synergistically suppress the proliferation of BoMacs without causing overt cytotoxicity (Oh et al., 2012). Three controls were included in the study; ‘DMSO control’ (containing both lipopolysaccharide (LPS) and DMSO equivalent to the highest treatment concentration), ‘control’ (containing only LPS), and ‘No LPS’ control (not containing neither LPS nor DMSO). These IC25s were chosen so the mycotoxins would inhibit cell proliferation without inducing cell death (Oh et al., 2012). For all treatments, cells were incubated for 6 h following mycotoxin exposure. This procedure was repeated three times for a total of three independent trials.

4.3.4. Total RNA isolation and cDNA synthesis

Total RNA extraction and isolation from the BoMacs was carried out using TRIZol reagent (Invitrogen, Canada) according to the manufacturer’s protocol. Total RNA isolated and RNA integrity number (RIN) was assessed with an Agilent 2100a Bioanalyzer (Agilent Tech Inc., U.S.A). Samples with a RIN value higher than 5 were used to synthesize cDNA. Reverse transcription of total RNA into cDNA was completed using Superscript III Reverse Transcriptase (Invitrogen, Canada) according to the manufacturer’s instructions. Each reaction used 500 ng of oligio (dT)12-18 primers (Invitrogen, Canada) and 1 μg of total RNA. Since the trials were repeated at least three different times, the extraction was performed separately for each trial.

4.3.5. Real-time PCR quantification

Target gene expression was evaluated with quantitative PCR (qPCR) by standard curve analysis (Rutledge and Côté, 2003) using the ABI Prism 7000 Sequence Detection System and ABI Prism 7000 SDS Software (Applied Biosystems, Canada). The qPCR was performed in a 25 μL reaction using Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen, Canada).
The qPCR protocol was 50 °C for 2 min, 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15s, annealing at primer-specific temperature for 30 seconds, and extension at 72 °C for 30s. A dissociation curve was run following each qPCR to ensure the presence of a uniform and single PCR product. Each cDNA sample from each trial was run in triplicate for each gene, and these triplicates were averaged to give mean of cDNA sample for each trial or block for the statistical analysis. Primer sequences, product size, and annealing temperatures are shown in Table 4.1.

4.3.6. qPCR amplification curve and statistical analyses

Cycle threshold (Ct) values for each sample were determined using the auto Ct function of the ABI Prism 7000 SDS Software; the Ct values were averaged between the three sample replicates. To derive input values, gene-specific standard curves were developed using pooled cDNA. The constitutively expressed housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to standardize the expression of the target genes and determine their relative expression. Data from the experiment was analyzed as a randomized complete block design, in which the trials represented the blocks, and the treatments consisted of two factors, OTA (with versus without) and other treatments (DMSO control, CIT, MPA, PA, PAT) in a factorial arrangement. The analysis was performed using the Mixed procedure from SAS 9.3. Preliminary residual plots indicated the standard deviation of gene expression increased with the mean for all the genes, and therefore all data was log-transformed prior to analysis in order to stabilize variances. Differences between treatment means were assessed using the Dunnett test, comparing the mean of the treatment with and without OTA to all other means for genes with a significant interaction, and DMSO control to each of CIT, PAT, MPA, PA, for genes without a significant interaction. A separate F-test was done for the comparison between ‘control’ and ‘No LPS’ to determine the
effect of LPS on the gene expression. (*) and (**) were denoted as significant differences at p-value < 0.05 and 0.01 relative to the control, respectively, and trends ≤ 0.10 were also noted.

4.4. Result

The effect of in vitro PM treatment on the relative expression of genes responsible for epigenetic modifications was evaluated in BoMacs using GAPDH as reference gene (Figure 4.1). LPS had no effect on the gene expression of the methylating and demethylating enzymes. LPS slightly induced the expression of Bmi-1 as well as HDAC-1, -2 and -3; however, the increase was not statistically significant (data not shown).

The expression of JMJD-3 was significantly induced by PA (p < 0.05; Figure 4.1E), and the expression of HDAC-3 was slightly suppressed by MPA (p = 0.1) and PA (p=0.08) (Figure 4.2C). The individual mycotoxin treatments appeared to alter HDAC-2 expression, yet the statistical test failed to detect the significance among the treatments due to high variance (Figure 4.2B). The expression of DNMT-1, DNMT-2, UTX (Figure 4.1A, B, E) as well as HDAC-1, HDAC-2 and Bmi-1 (Figure 4.2A, B and D) were not significantly affected by any of individual mycotoxin treatments at their respective IC25s.

When BoMacs were co-exposed to OTA and CIT, PAT, MPA, or PA, the relative expression of DNMT-3a, DNMT-3b, JMJD-3, and HDAC-3 was significantly affected by at least one of the PM treatments (Figure 4.1C, D and E). Although PA alone did not significantly affect the expression of DNMT-3b (p = 0.2) and HDAC-3 (p = 0.08), PA when combined with OTA showed a significant synergistic suppressive effect on the expression of both DNMT-3b (p < 0.05; Figure
4.1D) and HDAC-3 was observed (p < 0.01; Figure 4.2C). Likewise, the combination of CIT and OTA synergistically suppressed the gene expression of DNMT-3a (p < 0.01), DNMT-3b (p < 0.01), JMJD-3 (p < 0.01) as well as HDAC-3 (p < 0.05). When PAT was combined with OTA, it also synergistically down-regulated the expression of both DNMT-3a (p < 0.05; Figure 4.1C) and HDAC-3 (p < 0.01; Figure 4.2C). Lastly, the combination of MPA and OTA synergistically suppressed HDAC-3 expression (p < 0.05; Figure 4.2C). Combinations of mycotoxins appeared to alter HDAC-2 expression, but the difference was not statistically significant among the treatments due to high variance (Figure 4.2B). Lastly, the expression of DNMT-1, DNMT-2, UTX (Figure 4.1A, B, E) as well as HDAC-1, HDAC-2 and Bmi-1 (Figure 4.2A, B and D) were not significantly affected by OTA combined mycotoxin treatments.

4.5. Discussion

Previous studies have determined cellular and functional changes in macrophages following exposure to PMs (Oh et al., 2012; Sorenson et al., 1985, 1986), and some of these mycotoxins, such as OTA, have been proposed to alter various cell activities through epigenetic mechanisms (Marin-Kuan et al., 2008). The normal functions of macrophages, such as proliferation and inflammatory response, could indirectly be modulated via altered epigenetic patterns following mycotoxin exposure (Bowdrige and Gause, 2010; Ishii et al., 2009; Marin-Kuan et al., 2007).

In a previous study, dose-response relationships were determined for the following PMs: CIT, OTA, PAT, MPA, and PA using BoMac proliferation and cell death as endpoints (Oh et al., 2012). Based on this previous study, IC25s were selected for the current study to standardize mycotoxin exposure concentrations to a constant physiological response (e.g. cell proliferation) without
causing overt cytotoxicity. Environmental concentrations of these mycotoxins could potentially exceed the IC25s used in this study when the favorable conditions are provided to mold for mycotoxin synthesis. For example, both OTA and PA have been found at higher concentrations in maize silage (Müller and Amend, 1997; Reyes-Velázquez et al., 2008). Furthermore, PAT and MPA are sometimes found at similar or higher concentrations in silage (Mansfield et al., 2008; Schneweis et al., 2000). Although the IC25s of the mycotoxins used in the study, such as OTA, were higher than circulating concentrations reported from in vivo studies (Blank et al., 2003; Höhler et al., 1999), there is the potential for their bioaccumulation in certain target tissues, such as kidney, intestine, and cutaneous fat (Galtier et al., 1981; Prelusky et al., 1986), as well as their synergistic and additive interaction among other mycotoxins within these tissues (Sansing et al., 1976).

In the present study, alterations in the gene expression of key enzymes responsible for epigenetic regulation, such as DNMTs, JMJD-3, UTX, Bmi-1 and HDACs were measured in BoMacs exposed to the PMs. Lipopolysaccharide (LPS) was used to stimulate BoMacs prior to mycotoxin exposure to induce the enzymes, specifically the expression of HDAC-3 (Zhu et al., 2010), and to determine how the mycotoxins alter the LPS-induced gene expression of the enzymes. In this study, however, LPS did not significantly alter the expression of methylation and demethylation enzymes, though this dose has been previously shown to stimulate macrophage gene expression (Martinet et al., 1988). Lipopolysaccharide slightly induced the expression of Bmi-1 as well as all HDACs, but the increase was not significant (data not shown). Despite this, we were still able to assess the effects of mycotoxins on the basal expression of genes used this study, and expression of genes coding some of the epigenetic enzymes was determined to be significantly altered by PM exposure, especially by combinations of mycotoxins with OTA.
PA significantly increased the gene expression of the histone demethylase *JMJD-3*, which is involved in decreasing histone methylation that leads to up-regulation of certain target genes. For example, JMJD-3 demethylates lysine 27 in histone 3 (H3K27) at the p16^INK4A/p19^ARF locus, which inhibits cell-cycle progression of human diploid fibroblasts (Agger et al., 2009). Both p16 and p19 are tumor suppressor genes that can induce p53 activity triggering the caspase-apoptotic pathway (Bardeesy et al., 2002; Schuler et al., 2000). p53 can also induce cell-cycle inhibitory protein p21 activity, which functions to suppress cell proliferation (Wilson et al., 2006; Wong et al., 2011). Bando et al., 2003 in contrast, reported that PA blocks Fas-induced apoptosis of Burkitt's lymphoma Raji cells, maintaining cell viability. PA concentrations greater than 100 μM, however, significantly increased the cell cytotoxicity in both lymphoma Raji cells (Bando et al., 2003) and BoMacs (Oh et al., 2012). In present study, however, IC25s were chosen so that they would not cause overt cytotoxicity; the IC25 of 13.90 μM did not cause any cytotoxicity to the cells (Oh et al., 2012). This may be why the expression of *DNMT-1* and *DNMT-3s* was not significantly suppressed by PA (Figure 4.1D), since the activity of these two enzymes is crucial for cell survival (Beaulieu et al., 2002; Fatemi et al., 2002; Kurita et al., 2010).

PA also slightly inhibited the expression of *HDAC-3*, and so did MPA exposure (Figure 4.2C). HDAC-3 activity has been shown to be positively associated with the proliferation of both human colorectal adenocarcinoma cells and colorectal cancer cells (SW480); where inhibition of HDAC-3 activity increased cell-cycle inhibitory protein p21 in SW480, which inhibited cell proliferation (Spurling et al., 2008). Given this, it is possible that the inhibition of BoMac proliferation by either PA or MPA that was observed previously (Oh et al., 2012), could be partially due to the suppressed *HDAC-3* expression.
OTA is frequently detected in combination with either CIT or PA (Frisvad et al., 2004; Vrabcheva et al., 2000), and the combination of these mycotoxins has synergistic effects in various tissues, especially the liver and kidney (Sansing et al., 1976; Shepherd et al., 1981). PAT, on the other hand, is less commonly found with OTA as compared to CIT or PA, but both PAT and OTA are the common contaminants found in corn/maize silage (Mansfield et al., 2008; Reyes-Velázquez et al., 2008).

It is interesting to note that although CIT and OTA alone had no significant effect on gene expression, when combined, the expression of DNMT-3a, DNMT-3b, JMJD-3, and HDAC-3 was significantly reduced. Likewise, the combination of PA and OTA significantly suppressed the expression of DNMT-3b and HDAC-3. The other two PM combinations also showed synergistic suppression; PAT+OTA synergistically suppressed the expression of DNMT-3a and HDAC-3, while MPA+OTA synergistically down-regulated the expression of HDAC-3. These findings suggest that OTA when combined with any of other four mycotoxins, including CIT, PAT, MPA or PA, acts synergistically to alter the gene expression of these enzymes, especially HDAC-3 and this may lead to potential toxic effects that we reported previously with combinations of these PMs (Oh et al., 2012). These enzymes regulate de novo cytosine methylation patterns, specifically on H3K27 (Agger et al., 2009), which alters cell activities such as viability and proliferation.

The effects of mycotoxin exposure on DNMT expression could be important since DNMT-3a and DNMT-3b are known to methylate different sites within the genome (Hsieh, 1999; Okano et al., 1999). For example, they can both suppress p53 activity (Kurita et al., 2010; Wang et al., 2005), preventing activation of caspase apoptotic pathways (Schuler et al., 2000). In this study, CIT+OTA synergistically suppressed the expression of both DNMT-3a and DNMT-3b. PA+OTA suppressed the expression of DNMT-3b, while the combination of PAT+OTA inhibited the expression of
DNMT-3a. Inhibition of DNMT-3s may potentially induce p53-mediated programmed cell death (Kurita et al., 2010; Schuler et al., 2000; Wang et al., 2005). These combinations, however, failed to induce BoMac cell death in the previous cytotoxicity study (Oh et al., 2012). Kurita et al. 2010 reported that DNMT-1 and DNMT-3b functionally co-operate together to maintain cell viability, and silencing of both genes is required to induce programmed cell death (Kurita et al., 2010). Similar to DNMT-3b, DNMT-3a also functionally co-operates with DNMT-1 during de novo methylation (Fatemi et al., 2002). Therefore, it is possible that even though we observed suppressed expression of both DNMT-3s by CIT+OTA, DNMT3a by PAT+OTA, and DNMT-3b by PA+OTA in the present study, the suppression of DNMT-3s alone might not be sufficient to induce programmed cell death and/or inhibit cell proliferation (Wilson et al., 2006; Wong et al., 2011) as long as the expression of DNMT-1 is maintained.

The combination of PA and OTA significantly inhibited JMJD-3 expression that was originally induced by PA exposure alone. Inhibited JMJD-3 expression would likely induce cell proliferation (Wilson et al., 2006; Wong et al., 2011), yet our previous study determined that PA+OTA synergistically inhibited the proliferation of BoMacs (Oh et al., 2012). It is possible that other mechanisms or molecules that have similar function as JMJD-3 may help to maintain the activity that is typically carried out by JMJD-3 after its suppression by PA+OTA. For example, UTX is a paralog of JMJD-3 that is constitutively expressed, and has a similar function as JMJD-3 (Agger et al., 2007).

Co-exposure of OTA with either CIT, PAT, MPA, or PA synergistically decreased HDAC-3 expression. Given that the activity of HDAC-3 is positively correlated with cell proliferation (Spurling et al., 2008), the synergistic suppression in HDAC-3 expression by these mycotoxin combinations may lead to inhibited cell proliferation. In support of this, we previously observed
the significant synergistic suppression of BoMacs proliferation from most of these combinations, except for MPA+OTA (Oh et al., 2012), with the greatest synergistic suppression in both HDAC-3 expression and BoMacs proliferation being observed with the combination of PA and OTA. This suggests that the reduced expression of HDAC-3 by these mycotoxin combinations may potentially lead to the down-regulated HDAC-3 protein expression, and this may have contributed to the inhibited proliferation of BoMacs that was previously observed (Oh et al., 2012). HDAC-3 also regulates mitochondrial function and lipid metabolism (Sun et al., 2011), which are critical for the survival of kidney or liver cells (Begriche et al., 2011). Therefore, the synergistic inhibition of HDAC-3 expression by these mycotoxin combinations may also potentially contribute to the synergistic nephrotoxicity (Shepherd et al., 1981), and hepatotoxicity (Sansing et al., 1976) reported from the mice studies.

Although we did not find statistically significant changes in Bmi-1 expression by the mycotoxin treatments, a trend of suppression in Bmi-1 expression was observed with co-exposure of PA and OTA (Figure 4.2D), where the combination also synergistically suppressed HDAC-3 expression. Since Bmi-1 expression can be suppressed by histone deacetylase inhibitors (HDACi) in immortalized breast epithelial (MCF10A) cells (Bommi et al., 2010), these two enzymes could be involved with or regulated by the same molecular pathways. Similar to HDAC-3, Bmi-1 is a crucial epigenetic regulator of cell proliferation (Lessard and Sauvageau, 2003; Pietersen et al., 2008); Bmi-1 regulates cell-cycle progression, is important for DNA repair and the renewal of hematopoietic stem cells (Godlewski et al., 2008), and its expression is positively correlated with the growth of cancer cells (Qin et al., 2009). Bmi-1 was also reported to suppress p21 activity in mice cerebellar granule cell precursors (Subkhankulova et al., 2010). Both Bmi-1 and HDAC-3 regulate p21 activity that can alter cell proliferation. It is possible that the exposure duration was
not long enough to significantly affect *Bmi-1* expression, since cell proliferation would not have actively occurred within the exposure period. Furthermore, it is possible that higher concentrations of mycotoxins could have significantly altered the expression of *Bmi-1* and *HDAC-3*, even though this needs confirmation.

Regardless of the similar level of stress that BoMacs were subjected to by each mycotoxin, these mycotoxins at their IC25s differently modulated the relative gene expression of several epigenetic enzymes. It is possible that PMs inhibit cell proliferation via different pathways. For example, it has been proposed that MPA inhibits cell proliferation by inhibiting inosine monophosphate dehydrogenase (IMPDH) leading to the inhibition of *de-novo* purine biosynthesis (Wadia et al., 2009b). Since the present study only investigated the expression of mRNA, which may not necessarily reflect actual translation product and activity of epigenetic enzymes, it will be important in the future to measure protein expression as well as the activity of these enzymes in both *in vitro* and *in vivo* studies.

### 4.6. Summary and Conclusion

In summary, findings of the present study help to support the hypothesis that the consumption of food products contaminated with PMs, especially when combined, may affect the expression of epigenetic regulatory enzymes and this outcome could be a novel mechanism contributing to mycotoxicoses (Appendix 1). In future studies, it will be beneficial to study the effects of mycotoxin exposure on the expression of these genes using different physiological endpoints that are under the influence of epigenetic regulation, such as differentiation, apoptosis, or inflammatory response of macrophages. Additionally, it would be useful to explore the expression of genes coding other epigenetic enzymes such as UTX, as well as carry out global gene expression
profiling to identify candidate genes and gene pathways that may be influenced by these enzymes during \textit{in vivo} mycotoxin exposure.
Table 4.1: Summary of the designed oligonucleotide primer sequences (F forward primer, R reverse primer)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>PCR Product size (bp)</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
</table>
| DNMT-1   | F TCAGCGTGTACTGTAAACCGG  
           | R CATATATCGGCTTTTGCTGAACC | 102                    | 59                         |
| DNMT-2   | F ACCACAAAGTCATTACTGCGA  
           | R TGTCCCTTCTATGAGCGTCCA | 109                    | 56                         |
| DNMT-3a  | F ACCTTCCTGGTATGAAACAGCC  
           | R CCTCAGTTTGCTGAACCTTGCT | 104                    | 60                         |
| DNMT-3b  | F AGTGGTTTTGATGCGCAAGTT  
           | R ACCACCTTTGAGGCTCGCC | 103                    | 58                         |
| JMJD-3   | F TCTCGCCGGTGCTTTACAGTT  
           | R TGGAGAAGAGGGCCAAAGTTGAG | 102                    | 63                         |
| UTX      | F TGATCCCACGCTTTGTCGAG  
           | R AGCATTTGACAAAATGCGAG | 139                    | 56                         |
| Bmi-1    | F GAAATCTAAGGAGGAGGATGA  
           | R AGGTCAACTCTGATTTCAAGGG | 233                    | 59                         |
| HDAC-1   | F ACTCTTGAAACCCATATGGGTACTG  
           | R AATGGCTGATGGTACCTAACAG  
           | 180                    | 59                         |
| HDAC-2   | F TGGAGGATTACATCATGCTAACA  
           | R TATCCCTTAAGCTCCTGTTCCAG | 233                    | 59                         |
| HDAC-3   | F CAAGACTGTTGCTTTATTTCTACGA  
           | R TCAATGTAGTCCTCAGAGTGAAGG | 198                    | 59                         |
| GAPDH    | F TAATCTCTGTGCTGTGCCAGCC  
           | R TTTAAAGGCAGCCTGGTGACC | 103                    | 61                         |
Figure 4.1: (A-F) The effect of in vitro PM exposure on the expression of BoMac candidate methylating and demethylating genes relative to the GAPDH house-keeping gene. Relative expression is based on least square mean values for three independent trials. (*) and (**) denote significant differences at p<0.05 and 0.01 relative to the control, respectively.
Figure 4.2: (A-D) The Effect of *in vitro* PM treatment on the expression of BoMac candidate acetylating and deacetylating genes relative to the GAPDH. Relative expression is based on least square mean values for three independent trials. (*) and (**) denote significant differences at p<0.05 and 0.01 relative to the control, respectively.
Chapter 5: Effect of *Penicillium* Mycotoxins on Cytokine Gene Expression, Reactive Oxygen Species (ROS) production and Phagocytosis of Bovine Macrophage (BoMacs) Function

Submitted for publication: Oh S-Y, Mead PJ, Sharma BS, Quinton VM, Boermans HJ, Swamy HV LN, Smith TK, Karrow NA. *Toxicology*, submitted 2014 Nov 14th, 2014

5.1. Abstract

*Penicillium* mycotoxins (PMs) are natural contaminants that are commonly found in improperly stored animal feeds. Although exposure to certain PMs has been reported to affect immune function, little data are available for ruminant species. Therefore, in this study bovine macrophages (BoMacs) were exposed to the following PMs: citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA) and penicillic acid (PA), and macrophage function was assessed by measuring cytokine gene expression, the production of reactive oxygen species (ROS), as well as phagocytosis of *Mycobacterium avium* ssp. *Paratuberculosis* (MAP), the causative agent of Johne’s disease. Real-time PCR analysis of pro-inflammatory cytokine genes interleukin (IL)-1α and IL-6, anti-inflammatory cytokine genes IL-10 and transforming growth factor (TGF)-β, as well as the neutrophil stimulating cytokine genes IL-12 and IL-23 was assessed following 6 and 24 h of PM exposure at the concentration that inhibit BoMac proliferation by 25% (IC25). Mycotoxin treatments altered the expression of cytokine genes at 24 h. OTA significantly induced IL-1α expression (p < 0.05), while the expression of IL-6 was suppressed (p < 0.01). MPA significantly induced the expression of IL-1α (p < 0.05) and reduced the expression of IL-12α (p < 0.01) and IL-10 (p < 0.01). PAT significantly suppressed the expression of IL-23 (p < 0.01), IL-10 (p < 0.05) and TGF-β (p < 0.05). Neither CIT, nor PA affected the expression of these genes.
PMs did not affect BoMac intracellular ROS production and phagocytosis of MAP at their respective IC25, however, effects were observed when ROS and phagocytosis were assessed at higher sub-lethal concentrations. Pretreatment with PAT and PA for example, significantly decreased the percent phagocytosis of MAP at 5.0 µM (p < 0.01) and 15.6 µM (p < 0.01), respectively, but only PA significantly suppressed PAM-3 stimulated ROS production at 31.3 (p < 0.05) and 250.0 µM (p < 0.01). OTA did not affect ROS production, but it significantly increased the percent phagocytosis of MAP at 6.25 (p < 0.05) and 12.5 (p < 0.01). These findings suggest that exposure to sub-lethal concentrations of PMs can affect macrophage function, which could affect immunoregulation and innate disease resistance to pathogens.

5.2. Introduction

*Penicillium* molds are one of the most common mycotoxin-producing fungal species found in silage for animal feed (Almeida et al., 2000; Duong, 1996). Poor storage conditions for grains and silages often provide favorable environmental conditions, such as moderate temperature with high humidity, for mycotoxin production from these mold species (Mansfield et al., 2008). The *Penicillium* mycotoxins (PMs), such as citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA) and penicillic acids (PA), can frequently be found in animal feeds (Mansfield et al., 2008; Martins et al., 2002; Reyes-Velázquez et al., 2008), and are reported to have various toxicological effects when consumed by animals (Charoenpornsook et al., 1998; Dickens and Jones, 1965; Sansing et al., 1976). Some studies have also characterized PMs as immunomodulators that could predispose animals to secondary diseases (Ferrante et al., 2006; Islam et al., 2012).

A number of studies have used macrophages as an *in vitro* cell model to assess the potential effect of mycotoxins on immune system, (Ferrante et al., 2008; Islam et al., 2012; Sorenson et al.,
because they are one of the important innate immune cells that contribute to the host defense against pathogens (Wynn et al., 2013). Macrophages can function as host sentinel cells that recognize pathogens and initiate and regulate the host inflammatory response (Forman and Torres, 2002; Isomaki et al., 1996; Wynn et al., 2013). They also act as effector and regulatory cells that control pathogen invasion and the severity of the immune response by releasing various types of cytokines such as interleukin (IL)-1 and -6, as well as effector molecules such as reactive oxygen species (ROS) (Isomaki et al., 1996; Wynn et al., 2013). Macrophages can also regulate the subsequent acquired immune response that provides long-term protection against invading pathogens (Wynn et al., 2013; Xing et al., 2000). For example, macrophages secrete IL-12 during early *Mycobacterium bovis* infection (Xing et al., 2000), which favors interferon (IFN)-γ production that subsequently enhances macrophage effector function (Flynn et al., 1995). Therefore, an appropriate macrophage response is crucial for host survival (Segal, 2007), and failure to respond could potentially increase susceptibility to secondary infection (Ishikawa et al. 1998) and/or lead to autoimmune disease (Cavin et al., 2009).

Several studies have reported that exposure to PMs could potentially alter macrophage function, leading to an inappropriate innate and acquired immune response during infection (Ferrante et al., 2008; Islam et al., 2012; Johannessen et al., 2005). Most of these studies, however, have used primary cells or human or mice cell lines, even though livestock species, especially the ruminants, are often exposed to PMs via consumption of poorly stored silage (Mansfield et al., 2008). It is generally assumed that PMs are rapidly degraded by commensal microbes in the gut (Fuchs et al., 2008; Kiessling et al., 1984), however, their antimicrobial properties could attenuate microbial detoxification of these toxins.
Since ruminants are constantly exposed to a wide variety of pathogens such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne’s disease (Harris and Barletta, 2001; Momotani, 2012), it is reasonable to investigate the potential effect of PMs on bovine macrophage function and their interaction with MAP infection. Therefore, in the present study, the potential immunomodulatory effect of PMs on macrophage functions such as the expression of cytokine genes, ROS production, and phagocytosis of MAP was evaluated by using a bovine macrophage cell line (BoMacs) as an *in vitro* cell culture model.

5.3. Materials and Methods

5.3.1. Cell culture

BoMacs (Stabel and Stabel, 1995) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 2.0 mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS), 100 unit/ml of penicillin, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B, and 25 mM HEPES buffer. Cells were incubated at 37°C with 5% CO2 in 75 cm2 flasks, and reached at least 80% confluence prior to use for the study.

5.3.2. Cytokine gene expression

BoMacs (1.0 x 10^6) were seeded into six-well flat-bottom plates (Corning Inc., U.S.A), centrifuged for 2 min at 100 g, and incubated at 37°C with 5% CO2. After 1 h of incubation, the cells were stimulated with 1 μg/μl of *Escherichia coli* lipopolysaccharide (LPS; *E.coli* O111:B4, Sigma, Oakville, Canada) and exposed immediately to PM at their respective IC25 (the concentration that inhibit 25% of cell proliferation), which was determined from a previous study with BoMacs (Table 5.1; Oh et al., 2012). A total of five PM treatments (CIT, OTA, PAT, MPA, PA) were used, as well as three controls: +LPS/+dimethyl sulfoxide carrier solvent (DMSO
equivalent to the highest treatment concentration), +LPS/-DMSO and -LPS/-DMSO for each time point. All mycotoxins were ordered from Sigma-Aldrich, U.S.A. After 6 and 24 h of PM exposure, total RNA was extracted using TRIzol reagent (Invitrogen, Canada) according to the manufacturer’s protocol. Messenger RNA was then converted to cDNA, and the target gene expression was evaluated by semi-quantitative PCR (qPCR) using standard curve analysis and the PerfeCTa SYBR Green SuperMix (Quanta, BioScience Inc. U.S.A), and an ABI PRISM® 7000 (ABI applied Biosystem, U.S.A) (Table 5.2; Rutledge and Côté, 2003). The qPCR steps included: activation (50°C for 3 min), initial denaturing (95°C for 10 min), followed by a maximum of 40 cycles of denaturing (95°C for 30 s), annealing for 30 s (Temperatures in Table 5.2) and elongation (72°C for 30 s). Each trial was repeated 3 times to account for variability.

Three reference genes were tested, including β-actin, β-tublin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The expression of GAPDH was found to be the most stable across samples and treatments, therefore, GAPDH was used as a reference gene to standardize the expression of the following target genes and determine their relative expression (IL-1α, IL-6, IL-10, IL-12α, IL-12β, IL-23 and transforming growth factor (TGF)-β).

5.3.3. Measuring reactive oxygen species (ROS) and phagocytosis of MAP

BoMacs (1.0 x 10^5) were seeded into 96-black well flat-bottom plates, centrifuged for 2 min and incubated at 37°C with 5% CO₂. After 2 h of incubation, the cells were pre-exposed to series concentrations of PMs for 6 h (Table 5.1). For the ROS and phagocytosis studies, ethanol was used as solvent to dissolve all PMs, because DMSO was found to affect ROS production. Exposure media containing PMs was removed and cells were washed with incomplete media prior to either ROS or phagocytosis assay.
For ROS production, BoMacs were reconstituted in RPMI media containing 10.0 µl/ml Pam3CSK4 (PAM-3; ROS stimulator; InvivoGen, U.S.A) and 50 mM of Carboxy-H2DFFDA (5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (ROS indicator; Invitrogen, Canada). After 6 h of incubation at 37°C with 5% CO₂, the media was removed, cells were washed with PBS, and intracellular ROS production was immediately assessed by measuring intracellular fluorescing Carboxy-H2DFFDA residue (excitation 475 nm/ emission 510 nm) with a 1420 Victor 2 Multi-label Counter (PerkinElmer, U.S.A).

For phagocytosis of MAP, BoMacs were reconstituted in RPMI media containing mCherry-labeled MAP at multiplicity of infection (MOI) of 1:10, approximately 100,000 colony forming units (CFU) were used per well. The CFU of MAP was estimated by plotting the CFU versus optical density (OD) and OD versus mCherry fluorescence intensity, all of which were constructed previously in our lab (Qiumei, 2014). The plates containing both the cells and mCherry-MAP were centrifuged at 100 g for 5 min, and incubated for 6 h at 37°C with 5% CO₂. The media was then removed, cells were washed with RPMI media without FBS, and the amount of phagocytized mCherry-MAP was assessed by measuring mCherry fluorescence (excitation 587/ emission 610 nm) with a 1420 Victor 2 Multi-label Counter.

Cell viability was also measured after assessing both ROS production and phagocytosis of MAP by adding 20 µM Calcein AM (Invitrogen, Canada) to each well and incubating for 30 min at room temperature. Cell viability was assessed by measuring the fluorescence of intracellular Calcein AM (excitation 475 nm/ emission 510nm) using a 1420 Victor 2 Multi-label Counter. Although the fluorescence of Calcein AM overlaps with the Carboxy-H2DFFDA, the fluorescence of Carboxy-H2DFFDA contributed to less than 1% of Calcein AM fluorescence.
5.3.4. Statistical analysis

The gene expression data were analyzed as a randomized complete block design, in which the trials represented the blocks, and the treatments consisted of two factors, treatments (DMSO control, no LPS, control, CIT, OTA, PAT, MPA, PA) and time (6 and 24 h). The Mixed procedure (SAS 9.3) was used for statistical analysis. Preliminary residual plots indicated the standard deviation of gene expression increased with the mean for all the genes, and therefore all data were log-transformed prior to analysis in order to stabilize variances. Differences between treatment means were assessed using Dunnett’s test, comparing the mean of the treatment between 6 and 24 h to all other means for genes with a significant interaction, and DMSO control to each of CIT, PAT, MPA, PA, for genes without a significant interaction. (*) and (**) were denoted as significant differences at p-value < 0.05 and 0.01, respectively, relative to the DMSO control.

The ROS and phagocytosis of mCherry-MAP data were converted to percent comparison to the control using the following equation: \((T-NC)/(PC-NC)\), where \(T\) represents sample fluorescence reading, \(NC\) is the fluorescence reading of the negative control without PAM-3 stimulation or mCherry-MAP exposure, and \(PC\) is fluorescence reading of the positive control with PAM-3 stimulation or mCherry-MAP exposure but not the mycotoxins and ethanol. All the data were log transformed prior to statistical analysis. Brown and Forsythe tests of the transformed ROS production and phagocytosis data indicated variance differences among the toxins, so separate residual variances for each toxin were incorporated into the model prior to analysis. The Mixed procedure (SAS 9.3) was used for all analyses. The following data were analyzed as randomized complete block design, in which the trials represented the blocks. Cell viability, indicated as dotted line in Figures 5.2 and 5.3, and the interaction of cell viability and toxins was used as a covariate for all analysis. Dunnett’s test was used in all the analyses to compare each PM
concentration with its’ respective ethanol control on the same plate. (*) and (**) were denoted as significant differences at p-value < 0.05 and 0.01 relative to the ethanol control, respectively, and trends ≤ 0.10 were also noted.

5.4. Results

5.4.1. Effect of PMs on BoMacs cytokine gene expression

None of the PMs significantly affected cytokine gene expression at 6 h. Significant changes in cytokine gene expression were, however, evident within the treatments exposed to mycotoxins for 24 h. There was significant time (6 versus 24 h) and treatment (mycotoxins) interaction for the expression of both IL-1α and IL-6 (Figure 5.1A and B), but not for the expression of IL-10, IL-12α, IL-12β, IL-23, and TGF-β (Figure 5.1C-G). LPS significantly stimulated the gene expression of IL-6 at 24 h, but not at 6 h (p < 0.01; Figure 5.1B), and it also stimulated the expression of IL-12α (p < 0.01; Figure 5.1E). The increased expression of IL-12α was significantly attenuated by the DMSO carrier solvent (p < 0.01; Figure 5.1E), but DMSO had no effect on the gene expression of the other cytokines (Figure 5.1).

OTA at 24 h significantly induced IL-1α expression (p < 0.05; Figure 5.1A), and suppressed the expression of IL-6 induced by LPS (p < 0.01; Figure 5.1B). MPA also significantly induced the expression of IL-1α (p < 0.05; Figure 5.1A), but had no significant effect on IL-6 (Figure 5.1B). MPA also significantly reduced the expression of IL-10 (p < 0.01; Figure 5.1C) and IL-12α (p < 0.01; Figure 5.1E). The expression of IL-12β was slightly suppressed by MPA (p = 0.06; Figure 5.1F). PAT significantly suppressed the expression of both anti-inflammatory cytokines, IL-10 (p < 0.05; Figure 5.1C) and TGF-β (p < 0.05; Figure 5.1D) as well as IL-23 expression (p < 0.01; Figure 5.1G). CIT and PA did not significantly alter the gene expression of any cytokines, even though PA seemed to slightly reduce the expression of IL-23 (p = 0.07; Figure 5.1G).
5.4.2. Effects of PMs on BoMacs ROS production and phagocytosis of MAP

The potential effect of PMs on PAM3-stimulated ROS production as well as phagocytosis of mCherry-MAP was also investigated. Initial experiments indicated that the PMs at their respective IC25 did not affect these cell functions (Appendix 2). Therefore, a range of PM concentrations was tested to determine the effective of PM concentration after 6 h of exposure (Table 5.1).

The effect of these PMs on intracellular ROS production and percent phagocytosis of MAP was found to occur at much higher concentrations (Figure 5.2 and 5.3). OTA did not affect ROS production (Figure 5.2B), yet OTA at 6.3 μM (p < 0.05) and 12.5 μM (p < 0.01) significantly increased the percent phagocytosis of MAP (Figure 5.3B). PA significantly decreased the PAM-3 stimulated ROS production at the concentrations of 62.5 μM (p < 0.05) and 250.0 μM (p < 0.01; Figure 5.2E), and the percent phagocytosis of MAP was reduced at 15.6 μM (p < 0.01; Figure 5.3E). PAT did not affect ROS production, but showed decrease in the trend of ROS production throughout the concentrations tested (Figure 5.2C). PAT at concentrations greater than 2.5 μM also showed an inhibitory trend on the phagocytosis of MAP, and statistically inhibited phagocytosis at 5.0 μM (p < 0.01; Figure 5.3C). Neither CIT nor MPA significantly altered ROS production and percent phagocytosis of MAP (Figure 5.2A, D and 5.3A, D). All the significant effects observed from both ROS and phagocytosis studies occurred at the sub-lethal PM concentrations except for PA at 250.0 μM as shown with dotted line in Figures 5.2E and 5.3E.

5.5. Discussion

PMs are common contaminants found in food, especially in storage feed for animal consumption (Mansfield et al., 2008). Several studies have determined that PMs have immunomodulatory effects, altering the production of inflammatory cytokines from human and murine macrophages (Ferrante et al., 2006; Islam et al., 2012; Luft et al., 2008). The following
study also demonstrated that the PMs differently altered bovine macrophage cytokine gene expression as well as ROS production and phagocytosis, all of which are important for regulating inflammatory response and host resistance against bacterial infection.

Lipopolysaccharide (LPS) was used in the present gene expression study to stimulate BoMacs cytokine gene expression. LPS significantly increased the expression of \( IL-6 \), yet it did not significantly affect the expression of any other cytokines tested in the present study. Along with stimulated \( IL-6 \) expression, one would expect to also see an increase in \( IL-1\alpha \) expression in response to LPS stimulation, a well-known potent inducer of inflammation (Pang et al., 1994). A slight increase in \( IL-1\alpha \) expression was observed, but it was not statistically significant. This may be due to low TLR-4 expression associated with BoMacs, which is one of the main pattern-recognition receptors for LPS (Nomura et al., 2000). In a preliminary study, it was also noted that LPS failed to induce ROS production (Appendix 3), which was why PAM-3 was used as an alternate for LPS to stimulate ROS production.

DMSO, the solvent control, also appeared to significantly suppress the expression of \( IL-12\alpha \); however, it did not alter the expression of other cytokines. Since DMSO also affected both ROS production in preliminary study (data not shown), ethanol was used as a solvent to dissolve all PMs for the ROS production and phagocytosis assays.

The present study showed that OTA at its respective IC25 significantly induced \( IL-1\alpha \) expression after 24 h of exposure. IL-1\( \alpha \) is a potent pro-inflammatory cytokine that can induce the expression of other pro-inflammatory cytokines and molecules such as IL-6, hepatic acute-phase proteins, and the production of ROS (Dinarello, 1994). Interestingly, OTA did not significantly induce BoMacs \( IL-6 \) gene expression, but rather decreased it. Although IL-1\( \alpha \) and IL-6 are known to be potent inflammatory cytokines that stimulate macrophage ROS production (Radeke et al.,

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1990; Sung et al., 2000), IL-6 also possesses anti-inflammatory and regenerative capabilities that depend on its interaction with IL-6 receptors. Binding of IL-6 to soluble IL-6 receptors for example, induces ROS (Kojima et al., 2012), whereas when IL-6 interacts with membrane-bound IL-6 receptors, macrophages have an anti-inflammatory phenotype (Komohara et al., 2008). Therefore, the suppressed  *IL-6* expression observed in this study by OTA may not necessarily reflect increased and/or decreased inflammatory response of macrophages, and this is supported by the fact that ROS production was not induced even at higher OTA concentrations. Furthermore, these cytokines are stored as inactive pro-IL-1 and pro-IL-6 molecules in macrophage granules and become activated by caspase-1 (Maelfait et al., 2008). Therefore, it is likely that the expression of both *IL-1α* and *IL-6* in the present study does not reflect what is happening at the level of protein expression.

Higher concentrations of OTA (12.5 μM and 25.0 μM) in the present study also significantly increased BoMacs phagocytosis of mCherry-MAP, which from a host’s perspective could be good or bad, depending on the state of macrophage activation (Flynn et al., 1995; O’Leary et al., 2011). Interestingly, an acute exposure study carried out by Hibi et al. (2013) recently demonstrated significant induction of CD44 mRNA expression in rodent proximal tubule cells following OTA exposure; CD44 is also expressed on macrophages, and has been reported a major attachment site for *M. tuberculosis* (Leemans et al., 2003; Palaniappan et al., 2012). It would be interesting in future studies to assess if CD44 expression is altered on BoMacs by OTA exposure, and if this mediates phagocytosis of MAP.

Although no studies have previously investigated the effect of OTA on macrophage phagocytosis of MAP, previous *in vivo* studies using sheep red blood cells (sRBCs) as a target reported that OTA suppressed macrophage phagocytosis (Harvey et al., 1992; Hassan et al., 2012).
These contrasting results may be due to differences in study conditions (*in vitro* versus *in vivo*), the expression of receptors mediating phagocytosis (Harvey et al., 1992; Restrepo et al., 2014), and to the phagocytosis target, which in our case was an intracellular pathogen that relies on host phagocytosis for its survival and replication.

MPA was another PM that induced *IL-1α* expression. This was not anticipated, however, because MPA is a potent immunosuppressant that is used to prevent tissue rejection after organ transplantation and treating inflammatory diseases (Cohn et al., 1999; Weber et al., 1998). The results from this study, including up-regulated *IL-1α* expression and down-regulated *IL-10* expression, suggest that MPA induces an inflammatory response from BoMacs. This has also been previously observed from a couple of *in vivo* studies, where MPA significantly elevated the protein expression of IL-6, TNF-α and MIP-2 (Dzidic et al., 2010; Rand et al., 2005). It has also been reported that MPA suppressed the cell-mediated immune response by down-regulating the gene expression of *IL-12* and *IL-23*, all of which are important for cell-mediated immune response (Park and Scott, 2001; Uhlig et al., 2006). Regardless of its significant effect on cytokine gene expression, MPA, even at higher concentrations, did not affect ROS production or macrophage phagocytosis of MAP.

In contrast to these findings, a few studies have demonstrated that MPA is a potent suppressor of ROS production (Park et al., 2004, 2008). Park et al. for example, (2004; 2008) reported that MPA significantly inhibited ROS production by down-regulating the extracellular regulated kinase (ERK)1/2-mitogen-activated protein kinase (MAPK) mediated pathway (Svegliati et al., 2005; Yu et al., 2011). Since PAM-3 in the present study activated macrophage ROS production through TLR 1/2, which leads to nuclear factor (NF)-κβ signaling in addition to ERK1/2-MAPK signaling (Maelfait et al., 2008), it is possible that macrophages were able to maintain ROS production
regardless of MPA exposure concentrations. This is just a speculation, but warrants investigation in the future studies.

PAT did not alter the expression of \(IL-1\alpha\) and \(IL-6\), yet it significantly decreased the expression of \(IL-10\) and \(TGF-\beta\) in this study. Since IL-10 and TGF-\(\beta\) are considered anti-inflammatory cytokines (Isomaki et al., 1996; Paglinawan et al., 2003) and are important for steering the differentiation of regulatory T-cells (Taylor et al., 2006), their inhibition could favor cell-mediated immunity and excessive inflammation (Jarry et al., 2011).

PAT also significantly reduced the expression of \(IL-23\), which is an important cytokine for activating neutrophils (Mus et al., 2010; Pelletier et al., 2010), and promotes intestinal inflammation (Uhlig et al., 2006). Indramohan et al., (2012) also showed that IL-23 promotes the trafficking of monocytes to \textit{Listeria monocytogenes} infection sites, and regulates macrophage activity in an autocrine manner (Indramohan et al., 2014). Therefore, the suppression of \(IL-23\) expression by PAT could potentially reduce the functional activity of cell-mediate immune cells, such as the generation of ROS and macrophage phagocytosis (Mikawa et al., 1994). In the present study, PAT did not significantly affect ROS production, however, a trend of decreasing ROS production was noted at concentrations between 1.4 and 10.0 \(\mu\)M. Bourdiol et al. (1990) observed that 13 \(\mu\)M of PAT pre-incubation for 2 h significantly reduced superoxide anion production from murine peritoneal macrophages without affecting the cell viability. They also observed significantly inhibited ROS production at 26 \(\mu\)M, though this was probably due to decreased macrophage viability, because we observed that PAT dramatically decreased macrophage viability above 20 \(\mu\)M.

The present study also showed that PAT significantly decreased the phagocytosis of mCherry-MAP at 5.0 \(\mu\)M. In agreement with this study, Borudiol et al. (1990) reported that PAT
concentrations higher than 3.2 μM significantly decreased the phagocytosis of *Saccharomyces cerevisiae*. Additionally, their study found that the phagosome-lysosome fusion decreased above 0.65 μM, and lysosomal enzyme and microbicidal activity, and intracellular killing of yeast decreased at PAT concentrations above 3.2 μM (Bourdiol et al., 1990). Since the fusion of phagosome and lysosome is crucial for clearing MAP (Woo and Czuprynski, 2008), it is possible that PAT exposure could increase MAP survival during macrophage infection, and this warrants future investigation.

Similar to PAT, PA slightly reduced the expression of IL-23 (p = 0.07), and significantly suppressed ROS production at the concentrations between 31.3 μM and 125.0 μM. PA also significantly inhibited the macrophage phagocytosis of mCherry-MAP at 15.6 μM. We are unaware of any previous studies that have investigated the effect of PA on macrophage function. However, it appears that PA may share a similar toxic effect with PAT, based on the results from this study. Interestingly, Indramohan et al. (2014) reported a reduction of both ROS production and phagocytosis of *L. monocytogenes* in IL-23 knockdown mice as compared to normal C57BL/6 mice (Indramohan et al., 2014), This suggests that deceased IL-23 expression in the present study may have contributed to the observed decreases in BoMacs ROS production and phagocytic activity.

5.6. Summary and Conclusion

Changes in cytokine gene expression regulating the inflammatory and cell-mediated immune response were measured in BoMacs that were exposed to CIT, OTA, PAT, MPA or PA. OTA, PAT and PA also significantly affected macrophage ROS production and phagocytosis of the intracellular pathogen, MAP, at much higher sub-lethal concentrations. These results suggest that
sub-lethal concentrations of PMs can affect macrophage functions, which could in turn affect host resistance to intracellular pathogens.
Table 5.1: Exposure concentrations of PMs used in the cytokine gene expression, ROS and phagocytosis assay. IC25 values, determined from a previous study (Oh et al., 2012), were used for the cytokine gene expression study.

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>IC25s for gene expression study (Oh et al., 2012)</th>
<th>Concentrations used for the PAM-3 stimulated ROS production assay</th>
<th>Concentrations used for the phagocytosis of mCherry-MAP assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIT</td>
<td>52.7</td>
<td>18.8, 37.5, 75.0, 150.0, 300.0, 600.0</td>
<td>9.4, 18.8, 37.5, 75.0, 150.0, 300.0</td>
</tr>
<tr>
<td>OTA</td>
<td>8.9</td>
<td>1.6, 3.1, 6.3, 12.5, 25.0, 50.0</td>
<td>0.8, 1.6, 3.1, 6.3, 12.5, 25.0</td>
</tr>
<tr>
<td>PAT</td>
<td>0.3</td>
<td>1.3, 2.5, 5.0, 10.0, 20.0, 40.0</td>
<td>0.3, 0.6, 1.3, 2.5, 5.0, 10.0</td>
</tr>
<tr>
<td>MPA</td>
<td>0.5</td>
<td>28.1, 56.3, 112.5, 225.0, 450.0, 900.0</td>
<td>14.1, 28.1, 56.3, 112.5, 225.0, 450.0</td>
</tr>
<tr>
<td>PA</td>
<td>13.9</td>
<td>31.3, 62.5, 125.0, 250.0, 500.0, 1000.0</td>
<td>3.9, 7.8, 15.6, 31.3, 62.5, 125.0</td>
</tr>
</tbody>
</table>
Table 5.2: Summary of designed oligonucleotide primer sequences for cytokine gene expression study (F forward primer, R reverse primer).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>PCR Product size (bp)</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>F TCCATACCTGACGGCTACTACAT</td>
<td>138</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>R TGAGCACTCAAAACAGTTGAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>F CAGAGAAACCGAAGCTCTCAT</td>
<td>141</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>R CCTCCATTTTTGGGAAGATTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>F AAGGCCATGAGTGAGTTGACA</td>
<td>155</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>R TGGATTGGATTTCAGAGGTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-β</td>
<td>F CTGTGTTTCGTCAGCTCTACATT</td>
<td>129</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>R TACTGTGTATCCAGGCTCCAGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12α</td>
<td>F TGATGGATCCTAAAGGCAAAT</td>
<td>159</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R GAAGGATGCAGAGCTTTGACTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12β</td>
<td>F CTCTGAGTCGTCTCCTCCTCTT</td>
<td>127</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>R CCAGAAATAATCCTTTGCCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-23</td>
<td>F AGCAACTCTGAGCCCTAAGG</td>
<td>196</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>R TCAGCCTCTCCTAGGTCACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F GATGAGATTGGCATGGCTTTA</td>
<td>140</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>R AGAACCTTTGGAATGCTCGAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-tubulin</td>
<td>FATCATGAACACCTTTAGCGTC</td>
<td>114</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>RTAGGGTCTCCTCTCGTTTCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F ACCTGTCTGTTGAGATCGAC</td>
<td>112</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>RTAGCCTAGAAATGCCTTTGAGAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.1 (A-G): Effect of *in vitro* PM exposure on BoMac cytokine gene expression.
Figure 5.2 (A-E): Effect of 6 h in vitro PM exposure on BoMac ROS production. Exposure concentrations are listed in Table 1. Cell viability (represented as dotted line) was used as covariance for statistical analysis. 'contrl' on y-axis designated as a dotted line indicates the percent solvent (ethanol) control relative to controls without ethanol exposure. (*) and (**) denote significant differences at p<0.05 and 0.01 relative to the ethanol control, respectively.
Figure 5.3 (A-E): Effect of 6 h in vitro PM exposure on BoMacs phagocytosis of mCherry-MAP. Exposure concentrations are listed in Table 1. Cell viability (represented as dotted line) was used as covariance for statistical analysis. 'contrl' on y-axis designated as a dotted line indicates the percent solvent (ethanol) control relative to controls without ethanol exposure. (*) and (**) denote significant differences at p<0.05 and 0.01 relative to the ethanol control, respectively.
Chapter 6: In vitro Exposure of Penicillium Mycotoxins (PMs) with or without Mycosorb A+ (MA+) on Bovine Macrophage proliferation (BoMacs)

6.1. Abstract

Penicillium mycotoxins (PMs) are contaminants that are frequently found in grain or crop-based silage for animal feed. Previously, we have characterized the potential immunotoxicity of the following PMs: citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA) and penicillic acid (PA) by using a bovine macrophage cell line (BoMacs). In the present study, cell proliferation was used as a bioassay endpoint to evaluate the efficacy of Mycosorb A+ (MA+) which contains an esterified-glucomannan derived from a yeast cell wall component and blue-green algae, for preventing PM toxicity under various in vitro conditions such as: pH (3, 5, 7), incubation time (1, 2, 4, 6 h), percentage of MA+ (0.05, 0.1, 0.2, 0.5, 1.0%) and PM concentration. MA+ was most effective in preventing the toxicity of 12.88 µM and 25.8 µM OTA at pH 3.0 (p < 0.0001), regardless of incubation time (p < 0.0001) and the percentage of MA+ (p < 0.0001). An incubation time of 6 h (p < 0.05), or 0.5 and 1.0 percent MA+ (p < 0.0001) significantly improved the efficacy of MA+ for preventing CIT toxicity. In contrast, 0.5 and 1.0% of MA+ appeared to exacerbate the PAT toxicity (p < 0.0001). This exacerbated effect on PAT toxicity was constantly observed with higher PAT concentrations, and it reached significance at a concentration of 0.70 µM (p < 0.0001). MA+ had no effect on PA toxicity. These results suggest that MA+ may reduce OTA toxicity and to some extent CIT toxicity at pH 3.0. Although PAT toxicity was increased by MA+ treatment, PAT is readily degraded during heat treatment, and may therefore be dealt with using other preventative measures.
6.2. Introduction

Mycotoxins are natural toxicants produced by fungi that are commonly found in crops and stored grain-based animal feed (Boutrif, 1995). Previous studies have shown potential toxicity of mycotoxins when consumed by animals (Braunberg et al., 1992; Sansing et al., 1976). The consumption of animal feed heavily contaminated with mycotoxins can decrease livestock productivity and health, and this contributes to economic losses for producers (Schmale III and Munkvold, 2009). Due to their significant impact on agricultural commodities, many efforts have been made to reduce mycotoxin exposure via animal feed.

Although there are approximately 300 compounds that have been identified as mycotoxins (Bennett and Klich, 2003), studies have focused on only a few mycotoxins that have a history of outbreak, such as aflatoxins, T-2 toxins and deoxynivalenol (Forgacs, 1962; Marasas et al., 1987; Ramakrishna et al., 1989). In comparison to these Fusarium or Aspergillus mycotoxins, there are considerably fewer studies available related to mycotoxins produced from Penicillium spp., even though they are regarded as the most common mycotoxins found in silage for animal feed (Mansfield et al., 2008). Therefore, there is a need for more studies to examine their potential toxicity, as well as effective preventive methods to deal with Penicillium mycotoxins (PMs).

In previous studies, we used the bovine macrophage cell line (BoMacs) as an in vitro model to assess the potential toxic effects of the following PMs: citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA) and penicillic acid (PA). Endpoints including cell proliferation, viability, cytokine gene expression, reactive oxygen production and phagocytosis of Mycobacterium avium spp. Paratuberculosis (MAP) were used to assess PM immunotoxicity and/or immunomodulation (Oh et al., 2012, 2013). These studies demonstrated that these PMs
differentially modulate macrophage functions, which in turn could predispose animals to secondary disease.

The present study was designed to test the efficacy of the Mycosorb A+ (MA+; Alltech Inc. Kentucky) derived from the cell wall of *Saccharomyces cerevisiae* and culture extract of blue green algae (*Spirulina* spp.) for preventing the toxic effects of *Penicillium* mycotoxins using an *in vitro* BoMac proliferation bioassay. The objective of this study was to determine the optimal *in vitro* PM binding conditions, such as pH, incubation time and percentage of MA+ and PM concentration.

6.3. Methods
6.3.1. Preparation of cells

The BoMacs (provided by Stabel and Stabel, 1995) were cultured in Roswell Park Memorial Institute (RPMI) in 1640 medium, supplemented with 2.0 mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS), 100 unit/ml of penicillin, 100 µg/ml of streptomycin, 0.25µg/ml of amphotericin B, and 25 mM HEPES buffer. All cell culture products were purchased from Invitrogen, Canada. Cells were incubated at 37°C with 5% CO$_2$ in a 75 cm$^2$ flask, and were grown to above 80% confluence prior to use for the study.

6.3.2. Incubating PMs with MA+ and preparation of exposure media:

All mycotoxins were dissolved in ethanol for the study. The IC50s (The concentrations of PMs that inhibit 50% of BoMac proliferation) were selected as concentrations for testing the efficacy of MA+ based on a previous study (Oh et al., 2012). In this previous study, the IC50 for MPA could not be determined even at a concentration of 320 µM, where the solvent DMSO began
to inhibit cell proliferation. Therefore, 32 µM was used instead, because it was the highest MPA concentration previously tested that was not adversely affected by DMSO,. The IC50 of PAT was readjusted from 0.56 µM to 0.70 µM due to the decreased potency of PAT, probably due to the use of a different solvent. For these preliminary studies, one variable was addressed at a time, and a total of 4 different variables including pH, incubation time, percentage MA+, and various PM concentrations were considered. Mycosorb A+ was donated by Alltech Inc. for the purpose of this study.

To test the effect of pH, each PM was incubated with 0.2% MA+ in 500 ml of Milli-Q water at pH: 3, 5 and 7 for 2 h. The pH was adjusted with 1M of hydrogen chloride (HCl) and Sodium hydroxide (NaOH) after MA+ was added to Milli-Q water. To see the effect of incubation time, each mycotoxin was incubated with 0.2% MA+ in 500 ml of Milli-Q water at pH 3.0 for 1, 2, 4 or 6 h. To determine the most effective percentage of MA+ that inhibited mycotoxin toxicity, various percentages (0.05, 0.1, 0.2, 0.5, 1%) of MA+ were incubated with each mycotoxin in 500 ml of Milli-Q water for 2 h. Lastly, 0.2% MA+ was added to 5 different PM concentrations (Table 6.1) in 500 ml of Milli-Q water at pH 3 for 2 h to observe MA+ effectiveness at different PM concentrations. All incubations were carried out in 1.5 ml tubes that were placed horizontally at room temperature (22-25°C) with horizontal shaking at 30 g. After incubation, the MA+ was removed by centrifugation at 10,000 g for 10 min before the cell culture procedure. Supernatants containing the PM residues were then added to 96 well PCR plates for drying by desiccation for 24 h using silica gels & molecular sieves; Sigma-Aldrich, U.S.A) in a vacuum-sealed desiccator. After complete drying, residual PMs were reconstituted with RPMI media containing HEPES buffer (pH 7.2-7.4) prior to BoMacs culture.
6.3.3. BoMacs proliferation

BoMacs (1.0x10^4) were seeded into 96-well flat-bottom plates, centrifuged at 100 g for 2 min and incubated at 37°C with 5% CO₂ for 2 h until the cells adhered completely to the bottom of the plates. The media was removed and 100 µl of the prepared exposure media containing the PM residues was added to each well. After 47 h of PM exposure, a cell proliferation standard was prepared by seeding another batch of BoMacs in each plate (1.0x10^5, 7.5x10^4, 5.0x10^4, 2.5x10^4 and 1.0x10^3 cells per well), followed by centrifugation at 100 g for 2 min speed, then incubation for 1 h at 37°C with 5% CO₂.

The proliferation assay was carried out as described from a previous study (Oh et al. 2012). The media was removed from all wells by blotting, and the plates were placed in a -80°C freezer for at least 24 h to lyse the cells. The number of cells was estimated using a commercially available cell proliferation kit (Invitrogen, ON, Canada) that uses CyQUANT® GR dye (excitation 494/emission 517 nm) to label nucleic acids. The fluorescence intensity was measured with a 1420 Victor2 Multi-label Counter (PerkinElmer, U.S.A).

6.3.4. Data Analysis

All raw data were normalized to the number of cells based on a standard curve for each plate, and presented as percent cell proliferation compared to a negative control that contained MA+ but no PM. The percentage values for each treatment were log-transformed prior to statistical analysis in order to stabilize variances. The Tukey-Kramer test was used to compare the means of all the treatments that contained MA+ with the means of positive controls for each PM sample that contained mycotoxin but no MA+. For Figures 6.1 and 6.2, the positive control was also compared to another control that did not undergo air-dry to determine potential PM degradation during drying. For Figure 6.4, the dotted lines represent the positive control (PM without MA+), and solid lines
represent PM with MA+. (*), (**) and (***) denote significant differences at p < 0.05, 0.01 and 0.0001 relative to the positive control, respectively.

6.4. Results

The present study used the proliferation of BoMacs as a bioassay endpoint to determine the potential efficacy of MA+ for preventing PM toxicity. The drying procedure significantly reduced the toxicity of OTA (p < 0.05 in Figure 6.1B; p < 0.0001 in Figure 6.2B), but the efficacy of MA+ for OTA was still observed. MA+ was the most effective in preventing OTA toxicity and to some extent CIT toxicity among five mycotoxins tested in this study (Figure 6.1-6.4).

6.4.1. MA+ effectiveness at different pH levels

0.2% MA+ incubation for 2 h was effective in preventing OTA (p < 0.0001) and MPA (p < 0.0001) toxicity at pH 3.0 (Figure 6.1B and D). MA+ did not have a significant protective effect at pH 5.0 or 7.0.

6.4.2. MA+ effectiveness over time

A longer incubation time did not necessarily improve the efficacy of MA+ in preventing PM toxicity (Figure 6.2). MA+, however, showed a significant preventive effect on CIT toxicity (p < 0.01) by 9-33% after incubation for 6 h (Figure 6.2A). OTA toxicity was also significantly reduced by incubation with MA+ by 26-37% (p < 0.0001), but the incubation time did not necessarily improve its efficacy (Figure 6.2B).

6.4.3. Determining optimal effective percentage of MA+

As the percentage of MA+ increased, MA+ showed higher efficacy in preventing the toxicity of both CIT and OTA, but rather exacerbated PAT toxicity (Figure 6.3). The most effective MA+
percentage (1.0%; p < 0.0001) significantly protected against CIT-inhibited cell proliferation by 55-58% (Figure 6.3A). Furthermore, 1.0% MA+ protected against OTA-inhibited cell proliferation by 34-43% (p < 0.0001; Figure 6.3B). For PAT, MA+ did not reduce toxicity, but rather increased the toxicity at two highest percentages 0.5% and 1.0%, MA+ significantly increased PAT toxicity by 38-57% (p < 0.0001) and 45-57% (p < 0.0001), respectively. A separate viability test confirmed that this was due to increased cell death at these MA+ percentages (Appendix 4), even though MA+ alone had no effect on cell viability when used at 1.0% or below (Appendix 5). MA+ did not have a protective effect on MPA or PA toxicity regardless of the incubation time (Figure 6.2D and E) and the percentage of MA+ (Figure 6.3D and E). Although it was previously observed from the pH trials that 0.2% MA+ appear to have some degree of protective effect for MPA, this effect was not observed throughout other trials (Figure 6.2-6.4D).

6.4.4. MA+ efficacy at different PM concentrations

For the last study, five different concentrations of each PM were incubated with 0.2% of MA+ in Milli-Q water at pH 3.0 for 2 h (Figure 6.4). The study showed that the preventive efficacy of MA+ on PM toxicity largely dependent on PM concentration. MA+ significantly prevented OTA-inhibited cell proliferation with the most significant protection occurring at 12.88 µM by 38-45% (Figure 6.4B). In contrast, MA+ significantly increased MPA toxicity at 0.3 µM by 20-21% (p < 0.001; Figure 6.4D). MA+ also significantly exacerbated PAT toxicity at 0.7 µM by 36-71% (p < 0.0001; Figure 6.4C). In this study, MA+ did not significant affect PA toxicity (Figure 6.1-6.4E).

6.5. Discussion

In previous studies, we characterized the immunomodulatory effect of the following PMs, CIT, OTA, PAT, MPA and PA on macrophages using biological endpoints including proliferation,
cytotoxicity, ROS production and phagocytosis (Oh et al., 2012, 2013). Since BoMac proliferation was the only endpoint to be commonly affected by these PMs, it was chosen to test the efficacy of MA+.

The MA+ used in this study is a new variant of glucomannan mycotoxin adsorbent (GMA) sold commercially under the name of Mycosorb A+ by Alltech Inc. (Kentucky), and was derived from combinations of blue-green algae and a glucomannan-enriched cell wall component of *Saccharomyces cerevisiae*. Glucomannan reduces the bioavailability of mycotoxins by binding to the mycotoxins via hydrogen bonding and van der Waals force (Yiannikouris et al., 2004), and dietary inclusion of glucomannan added mycotoxin adsorbent product has become a popular post-harvest strategy to prevent mycotoxicoses in livestock (Kabak et al., 2006). Studies have shown glucomannan reduces toxic effects caused by aflatoxins and some *Fusarium* mycotoxins (Dvorska et al., 2007; Karaman et al., 2005; Korosteleva et al., 2007; Raju and Devegowda, 2000). Blue-green algae, such as *Spriulina spps.*, has been tested not only as a potential binder for aflatoxins, T-2 toxins and OTA but also as a potential anti-oxidant counteraction the oxidative stress from aflatoxins (Fanelli et al., 1985; Manafi et al., 2009, 2012). In comparison to these mycotoxins, there are only a few studies that have investigated the effectiveness of MA+ on PMs, and these have been limited to OTA (Evans and Dawson, 2000; Raju and Devegowda, 2002, 2000).

In the present study, MA+ was most effective at preventing the toxic effects of CIT and OTA among the five tested PMs. This finding is in agreement with studies done by Raju and Devegowda (2000, 2002), where they observed significant *in vitro* binding as well as the preventive effect of esterified-glucomannan on OTA toxicity to broiler chicks. Raju and Devegowda (2002), however, reported that pH did not alter the binding efficacy of the glucomannan at 2 mg/kg of OTA, where they observed 25% binding of OTA to the glucomannan at pH 4.5 and 6.5. In contrast, we observed
a huge effect of pH on MA+ effectiveness for preventing OTA toxicity, in which MA+ significantly reduced the toxicity of OTA up to 42% at pH 3.0, but not at either pH 5.0 or pH 7.0. Fruhauf et al., (2012) reported that mineral content, such as smecitite and calcite, is one of important factors that affected the binding of mycotoxins to yeast cell wall components. Since Raju and Devegowda (2002) used sodium phosphate buffer (PBS) when the esterified-glucomannan was incubated with OTA in vitro, whereas Milli-Q water, was used in the present study, this may explain the enhanced binding of OTA to the glucomannan in their study regardless of the pH difference.

The present study also showed that pH is a major factor that contributes to the efficacy of MA+ for preventing OTA toxicity. el Khoury and Atoui (2010) postulated that pH can alter the solubility of OTA. For example, OTA is soluble in polar organic solvents at acidic and neutral pH, whereas it is less soluble at high pH. Therefore, in the present study, OTA at pH 3.0 might have undergone a conformational change, which could have increased the strength of hydrogen bonding and van der Waals force between OTA and glucomannan component of MA+ in Milli-Q water during the incubation resulting in higher binding capacity (el Khoury and Atoui, 2010; Yiannikouris et al., 2004).

Although MA+ also significantly decreased the toxicity of MPA at pH 3 (Figure 6.1D), this was not reproducible in the other trials (Figure 6.2-6.4). This suggests that the combined effect of pH and drying might have affected the potency of MPA during the pH testing trials.

Incubation time was another factor that affected MA+ binding to PMs. For example, 0.2% MA+ protected the BoMacs from CIT toxicity only after 6 h of the incubation. In contrast, incubation of 0.2% MA+ at pH 3.0 in water effectively reduced OTA toxicity independent of the
incubation time (Figure 6.2B), whereas, it did not affect the toxicity of PAT, MPA and PA (Figure 6.2 C, D and E).

The recommended levels of GMA for commercial inclusion in animal feeds with a heavy mycotoxin contamination range from 0.05 % to 0.2%, which has been reported to have beneficial effects in reducing the signs of mycotoxicoses in livestock (Bursian et al., 2004; Evans and Dawson, 2000; Raju and Devegowda, 2002). For this study, different percentages of MA+, ranging from 0.05% to 1.0%, were tested to determine if different inclusion levels of MA+ would affect PM toxicity. This study showed that higher percentages of MA+ were more effective at preventing CIT and OTA toxicity than lower percentages. Similarly, Evans and Dawson (2000) also observed much higher binding of ergotamine with 0.2% Mycosorb®, another variant of GMA without blue-green algae from Alltech, as compared to that of 0.01% Mycosorb. This suggests that the percent level of glucomannan product in animal diets may contribute as more important than incubation time, at least for CIT and OTA, for reducing toxicity in vitro. This, however, would require confirmation through in vivo trials.

It should be noted that some of the PM concentrations used in this study were higher than those found in natural conditions (Garon et al., 2006; Müller and Amend, 1997; Reyes-Velázquez et al., 2008). Additionally, it is possible that high concentrations of PMs might have saturated MA+ binding sites. Therefore, different ranges of PM concentrations were also tested to determine if MA+ efficacy at 0.2% was dependent on PM concentrations. As expected, the efficacy of 0.2% MA+ for decreasing OTA toxicity was significant at 12.88 and 25.78 µM.

It was interesting to note that at low concentrations of CIT (11.40 and 22.80 µM) and MPA (0.03 and 0.32 µM), the incorporation of MA+ appeared to slightly increase the toxicity of these
two mycotoxins but not at higher concentrations. There is no clear explanation for this phenomena. In contrast to MPA and PA, CIT toxicity might have effectively been prevented if a longer incubation time, a higher amount of MA+, or a combination of the two were used together.

Regardless of its potential efficacy in preventing CIT and OTA toxicity, MA+ appeared to exacerbate PAT toxicity. For example, 0.1% MA+ appeared to slightly reduce PAT toxicity, but its beneficial effect was negated at 0.2% MA+ and PAT toxicity increased when incubated with 0.5% and 1.0% MA+ for 2 h. A similar observation was made when the various PAT concentrations were incubated with 0.2% MA+, and this reached statistical significant at 0.70 µM. Previously, Yue et al. (2011) tested 10 different strains of inactive yeast powder, and observed that at least 8 strains significantly reduced the concentration of PAT more than 50% in apple juice after 24 h of incubation at 25°C. Based on this previous binding study, one would expect a decrease in PAT toxicity to BoMacs. It is possible that soluble components of MA+, which could not be eliminated from the centrifugation method of this study, might have bound to PAT and protects it from degradation during the drying process. In fact, both Figures 6.1C and 6.2C indicates that the drying process slightly decreased the potency of PAT. Nevertheless, further study would be required to provide with more concrete explanations for this.

This is probably the first time a bioassay was used to test the efficacy of mycotoxin adsorbent for preventing toxic effects of PMs. The method allows us to look at the efficacy of MA+ in preventing the toxicity of PMs rather than looking at the binding efficacy of MA+. Therefore, it seems that this method reflects more of the bioavailability of mycotoxins as compared to other binding tests using HPLC (Evans and Dawson, 2000; Yue et al., 2011). However, the method cannot determine whether MA+ binds to PMs or not. Therefore, it would be important to do a follow-up study with HPLC-MS to understand how MA+ or other mycotoxin reducers work in
reducing the mycotoxicoses, and therefore, to design a better tool for reducing the bioavailability of mycotoxins to animals.

6.6. **Summary and Conclusion**

In the present study, we have shown that incubation time and pH as well as the amount of MA+ and the mycotoxin concentrations are the factors that can alter the efficacy of MA+ in preventing the toxicity of some PMs. As for OTA, pH was the major factor, while incubation time and amount of MA+ were the most important for preventing CIT toxicity. MA+ did not appear to effectively prevent the toxicity of PAT, MPA and PA in the present study, but rather increased the toxicity of PAT. Therefore, more research would be required to understand why MA+ increased PAT toxicity in the present study.
Table 6.1: Summary of experimental variables and constant factors for each Figure.

<table>
<thead>
<tr>
<th>Factors tested</th>
<th>Constant Factors</th>
<th>Experimental (Variable) Factors</th>
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<tbody>
<tr>
<td></td>
<td>Incubation Media</td>
<td>Temperature (°C)</td>
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<tr>
<td>Effect of pH (Figure 1)</td>
<td>Milli-Q Water</td>
<td>22-25°C with 150 rpm horizontal shaking</td>
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<td>Effect of incubation time (Figure 2)</td>
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<td>Effect of MA+ percentage (Figure 3)</td>
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<td>Different PM concentrations (Figure 4)</td>
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Figure 6.1 (A-E): The effect of pH (3, 5 and 7) on the efficacy of 0.2% MA+ in preventing the PM toxicity on BoMac proliferation.

The dotted line with 'control' label indicates negative control (control with MA+ incubation but without mycotoxin exposure) at 100% proliferation. (*), (**) and (***) denote significant differences at p < 0.05, 0.01 and 0.0001 relative to the control, respectively.
Figure 6.2 (A-E): The effect of incubation time (1, 2, 4 and 6 h) on the efficacy of 0.2% MA+ in preventing the PM toxicity on BoMac proliferation. The dotted line with 'control' label indicates negative control (control with MA+ incubation but without mycotoxin exposure) at 100% proliferation. (*), (**), and (***), denote significant differences at p < 0.05, 0.01 and 0.0001 relative to the control, respectively.
Figure 6.3 (A-E): The effect of percent amount of MA+ (0.05, 0.1, 0.2, 0.5 and 1 %) on the efficacy of MA+ in preventing the PM toxicity on BoMac proliferation. The dotted line with 'control' label indicates negative control (control with MA+ incubation but without mycotoxin exposure) at 100% proliferation. (*), (***) and (****) denote significant differences at p < 0.05, 0.01 and 0.0001 relative to the control, respectively.
Figure 6.4 (A-E): The effect of 0.2 % MA+ at different PM concentrations. The dotted plot line indicates the positive control of PMs without MA+ during incubation, and the solid plot line indicates the treatments that contained both PMs and MA+ during the 2 hrs of incubation. (*), (**) and (***) denote significant differences at p < 0.05, 0.01 and 0.0001 relative to the control, respectively.
Chapter 7: Assessing Interactions of Binary Mixtures of *Penicillium* Mycotoxins (PMs) by using Bovine Macrophage Cell Line (BoMacs) with the Assumption of Additivity Model

7.1. Abstract

*Penicillium* mycotoxins (PMs) are common contaminants found in stored silage for animal feed. Several studies have reported the potential toxicity of single PMs. However, this may not reflect the true risks associated with PMs, because PMs are often found in combinations of two or more, and therefore there are possible interactions, such as synergism or antagonism, among these PMs. We have previously demonstrated interactions between PMs in their ability to inhibit the proliferation of a bovine macrophage cell line (BoMacs). The study however, was lacking in both statistical and experimental methods for determining true interactions among the binary mixtures of PMs. Therefore, in the present study, we have re-examined the interactions of binary mixtures of PMs by applying the conceptual models of additivity, including independent action (IA) or concentration addition (CA), which are generally used to test mixture toxicity. When the previous IC25 data (concentration that inhibited the proliferation of BoMacs by 25%) were reanalyzed, the IA model determined that OTA combined with either CIT, PAT or PA demonstrated synergism (p < 0.05), while PA combined with either PAT or MPA showed antagonism (p < 0.05). The CA model determined that OTA+PA showed synergism (p < 0.05), while PAT+PA showed antagonism (p < 0.05). However, when a more complex experimental design was carried out using binary combinations of three different dilution levels (i.e. IC25, 0.5IC25, 0.25IC25) under the assumption of the IA model, CIT+OTA and PAT+PA showed synergism at the 0.5IC25s (p < 0.05) with a significant quadratic relationship occurring between the observed and expected effect (p < 0.0001). PAT+PA at their IC25s exhibited antagonism (p < 0.05), and PAT+MPA showed...
antagonistic interactions across all exposure levels. Other binary mixtures of PMs, including CIT+MPA, CIT+PA and MPA+PA, also showed antagonism at their IC25s and 0.25IC25s (p < 0.05) with a significant linear effect (p < 0.0001). There was no significant binary interaction observed at the 0.25IL25. The present study demonstrated that binary interactions among PMs are largely dependent on concentrations of the PMs. Furthermore, the results varied depending on which conceptual model was applied to the study. The present study provided an informative and fast initial screening to determine interactions of many binary PM mixtures, taking account of the variability of interactions of PM mixtures. Therefore, the study may be useful as an initial test in narrowing down some important mixtures of PMs with interactions prior to use of more complex experimental models, such as isobolograms or ray ratio design.

7.2. Introduction

The term 'mycotoxin' was first coined in 1962 following an outbreak on a turkey farm in London, England. Since then, mycotoxins have garnered much attention from the public and researchers for their potential to reduce both human and animal food safety (Bennett and Klich, 2003; Forgacs, 1962). Penicillium molds are one of the most common species of mycotoxin-producing fungi, and frequently contaminate crops and stored grains (Lennox et al., 2003). Penicillium mycotoxins (PMs) can lead to substantial economic loss to the animal feed industry by decreasing crop yields and value, as well as compromising livestock productivity and health (Schmale III and Munkvold, 2009).

PMs have been shown to cause a wide range of toxic effects in animals. Nephrotoxicity and hepatotoxicity are probably the most common clinical signs reported in animals fed PM contaminated feed (Braunberg et al., 1992; Dickens and Jones, 1965; Sansing et al., 1976). Some
studies have also reported their potential modulatory effect on the host’s immune response (Ferrante et al., 2006; Keblys et al., 2004; Stec et al., 2009). For example, Keblys et al., (2004) reported the dose-dependent inhibition of bovine lymphocyte proliferation by PMs, and a similar observation was made with porcine lymphocytes (Stec et al., 2009). We have previously reported significant inhibitory effects of the following PMs: citrinin (CIT), ochratoxin A (OTA), patulin (PAT), mycophenolic acid (MPA) and penicillic acid (PA), on the proliferation of a bovine macrophage cell line (BoMacs) (Oh et al., 2012).

Most toxicity studies involving mycotoxins have focused on the toxicity of individual mycotoxins. However, contaminated animal feeds in reality do not usually contain a single mycotoxin, but are rather contaminated with various combinations of mycotoxins. Mansfield et al. for example, reported that more than 50% of maize silage collected from 30 different Pennsylvania dairies between 2001-2002 contained more than one mycotoxin (Mansfield et al., 2008). Therefore, the potential risk of exposure to mycotoxins may be underestimated due to synergistic interactions, or overestimated due to antagonistic interactions among mycotoxins (Sansing et al., 1976; Shepherd et al., 1981; Veselá et al., 1983).

In the context of PMs, various interactions have also been reported. Specifically, CIT and PA toxicity was increased synergistically when animal co-exposure occurred with OTA (Sansing et al., 1976; Veselá et al., 1983). We have also determined from a previous study that binary PM mixtures at their respective IC25s (the concentration of PM that inhibited BoMac proliferation by 25%) also synergistically inhibited cell proliferation without causing overt cytotoxicity (Oh et al., 2012). This previous proliferation study, however, was lacking in both experimental and statistical methods for more precisely determining interactions between PMs. Specifically, conclusions were
solely based on single concentration data, and therefore did not take into account binary interactions at different PM concentrations (Meadows et al., 2002; Oh et al., 2012).

There are several different ways of determining the interaction of compounds in a mixture, and one common method is to use the assumption of additivity, whereby the compounds in the mixture are expected to have neutral risk when acting together (Berenbaum, 1981; Cedergreen et al., 2008; Stork et al., 2007). There are two basic concepts for predicting the combined effects of mixtures from the assumption of additivity: independent action (IA) and concentration addition (CA) (Cedergreen et al., 2008; Kjaerstad et al., 2010; Kortenkamp and Altenburger, 1998). The IA model assumes that compounds in a mixture have a completely independent mode of action that affects the same endpoint, while the CA model assumes that the compounds have a similar mode of action. In the absence of the mode of action of compounds in mixture, the IA model is often used as a default in assessing the mixture’s toxicity on human or animals, while the CA model is used as a default in environmental risk assessments (European Commission and Directorate General for Health & Consumers, 2012).

In the present study, the conceptual models were applied to re-examine the potential interaction of binary mixtures of PMs. The mode of action of these PMs on the proliferation of BoMacs is unknown at this point. Considering that the purpose of this study is to assess the toxicity of PMs on animals at dilutions of certain effect levels such as the mixture of IC25s, rather than an environmental assessment, the IA model was chosen over the CA model to estimate the additivity of PMs in the mixtures in the present study. In addition to this, the IA and CA models were also applied to the proliferation data from the previous study, and re-analyzed in order to compare the results from the present study.
7.3. Methods

7.3.1. Data Analysis and Plotting of the Previous PM Toxicity Data

The previous proliferation data (Oh et al., 2012) were analyzed and plotted to determine the interaction of the binary mixtures by using independent action (IA) or concentration addition (CA) models.

Based on the assumptions of the IA model, the effect of binary mycotoxin mixture was estimated from the observed effect for the individual mycotoxin exposure using the following equation (Jørgensen, 2013): \( P_{EM} = P_A \times P_B \), where \( P_{EM} \) represents the estimated percent proliferation from the mixture, and \( P_A \) and \( P_B \) represents the percent proliferation from the individual mycotoxins. The observed percent proliferation from the mixture (\( P_{OM} \)) and \( P_{EM} \) were plotted in Figure 7.1 to determine the interactions among the mycotoxins. For example, if the estimated proliferation fell within the 95% confidence limits (CI) of the observed proliferation, then the assumption of additivity was accepted, and mycotoxins within the mixture were said to have an additive effect. In contrast, when the mean of \( P_{EM} \) was outside of the 95% CI of \( P_{OM} \), then significant interaction between two mycotoxins occurred (\( p < 0.05 \)) as either synergism (\( P_{EM} \) being located above 95% CI of \( P_{OM} \)), or antagonism (\( P_{EM} \) being located below 95% CI of \( P_{OM} \)). Significant differences at a p-value \(< 0.05\) were denoted as (*) in Figure 7.1.

For the CA model, whole dose-response mixture prediction curves for the binary mixtures were constructed from the proliferation data of individual mycotoxin. The effect concentrations (ECs) for each proliferation response (eg. the concentrations that inhibited BoMacs proliferation by 99%...1%) were calculated from the curves giving the best fit, including biphasic curves (SigmaPlot 13). To calculate a mixture CA curve from several curves with different slopes, several
EC concentrations are calculated from fixed effect values. The mixture concentrations eliciting a certain effect are then calculated by $1/(p_A/EC_A+p_B/EC_B...p_n/EC_n)$, where A and B represent different mycotoxins with p being the proportion of the individual mycotoxin in the mixture ($\sum(p_A...p_n)=1$ (Ohlson et al., 2010). From the data, a theoretical (or prediction) curve for each PM mixture was constructed to compare with the observed proliferation. The error-bars are 95% CI of $P_{OM}$ (observed). For both the IA and CA model, if the prediction curve falls out of 95% CI of the ‘observed’ at a specific concentration, it indicates significant interaction between two PMs, and therefore, is defined as either synergism ($P_{EM}$ being located above 95% CI of $P_{OM}$) or antagonism ($P_{EM}$ being located below 95% CI of $P_{OM}$). (*) was used to denote significant differences at p-value < 0.05 in Figure 7.2.

7.3.2. Assessing the interactions of PMs using binary combinations over the dilutions of IC25s

7.3.2.1. Cell Preparation

The BoMacs (provided by Stabel and Stabel, 1995) were cultured in Roswell Park Memorial Institute (RPMI) in 1640 medium, supplemented with 2.0 mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS), 100 unit/ml of penicillin, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and 25 mM HEPES buffer. All cell culture products were purchased from Invitrogen, Canada. Cells were incubated at 37°C with 5% CO$_2$ in a 75 cm$^2$ flask, and were grown to above 80% confluence prior to use for the study.

7.3.2.2. BoMacs Exposure to Mycotoxins

BoMacs (1.0 x 10$^4$) were seeded into 96-well flat-bottom plates, centrifuged at 100 g for 2 min and incubated at 37°C with 5% CO$_2$ for 2 h until the cells completely adhered to the bottom of the plates. The BoMacs were then exposed to three dilutions (0, 0.5, 0.25) of the mycotoxin
concentration that inhibited BoMacs proliferation by 25% (IC25s) in either combinations or individual concentrations of CIT (52.72 μM), OTA (8.91 μM), PAT (0.32 μM), MPA (0.50 μM) and PA (13.90 μM) (Oh et al., 2012). All PMs were ordered from Sigma, Aldrich, U.S.A, and dissolved in ethanol prior to use. After 47 h of mycotoxin exposure, a cell proliferation standard was prepared by seeding another batch of BoMacs (1.0x10^5, 7.5x10^4, 5.0x10^4, 2.5x10^4 and 1.0x10^3 cells per well) in each plate, centrifuging at 100 g for 2 min, then incubating for 1 h at 37°C with 5% CO₂.

The proliferation assay was carried out as described previously (Oh et al. 2012). The media was removed from all wells by blotting, and the plates were frozen in a -80°C freezer for at least 24 h to lyse cells. The number of cells was estimated using a commercially available cell proliferation kit (Invitrogen, ON, Canada) that uses CyQUANT® GR dye (excitation 494/emission 517 nm) to label nucleic acids. The fluorescence intensity was measured with a 1420 Victor 2 Multilabel Counter (PerkinElmer, U.S.A).

7.3.2.3. Data and Statistical Analyses

All raw data were normalized to cell number based on the standard curve of each plate, and presented as percent cell proliferation compared to the control. Since the mode of action by which PMs inhibit BoMac proliferation is unknown, the independent action (IA) model was applied to estimate the additive effect of PMs on cell proliferation (European Commission and Directorate General for Health & Consumers, 2012).

In this study, two factors were statistically analysed. To test the linear or quadratic trends over dilution levels (IC25, 0.5IC25, 0.25IC25), linear and quadratic orthogonal polynomial contrasts were used between the plots of \( P_{OM} \) and \( P_{EM} \) (SAS 9.3; Kuehl, 2000). Similar to the previous IA model analysis, the difference between \( P_{OM} \) (indicated as ‘observed’) and \( P_{EM} \)
(indicated as ‘estimated’) for each respective dilution was evaluated using the 95% confidence interval (CI) of $P_{OM}$ in comparison to the mean of $P_{EM}$ as indicated in Figure 7.3.

### 7.4. Results

The proliferation data from the previous studies were re-analyzed and plotted by using a proper IA or CA model to determine the interactions among the mixtures of PMs. From the IA model, the mixtures of OTA with CIT, PAT, or PA showed significant synergism, while the mixtures of PA with PAT, or MPA showed significant antagonistic interactions on BoMac proliferation ($p < 0.05$; Figure 7.1). From the CA model, OTA+PA was the only mixture that showed a synergistic interaction ($p < 0.05$; Figure 7.2D), while PAT+PA showed an antagonistic interaction ($p < 0.05$; Figure 7.2I).

In the follow-up study, mixtures of three different dilutions were tested for each binary mixture of PMs. The mixture of IC25s, the first dilution of IC25 mixtures and the second dilution of IC25s are denoted as ‘IC25,’ ‘0.5IC25’ and ‘0.25IC25 in Figure 7.3, respectively. The study determined that some binary mixtures of PMs had significant interactions between $P_{OM}$ (observed) and $P_{EM}$ (estimated) with a presence of a significant linear or quadratic relationship. For example, CIT+OTA showed a significant difference in the quadratic relationship ($p < 0.01$), where the 0.5IC25 mixture of $P_{OM}$ exhibited significant synergistic inhibition of BoMac proliferation in comparison to that of $P_{EM}$ ($p < 0.05$), but not at the combinations of IC25 or 0.25IC25 (Figure 7.3A). PAT+PA also showed significant synergism at the mixture of 0.5IC25 ($p < 0.05$; Figure 7.3I). However, the mixture also exhibited significant antagonism at the mixture of their IC25s ($p < 0.05$) with a significant quadratic relationship between $P_{OM}$ and $P_{EM}$ ($p < 0.05$). Similar to PAT+PA, the mixture of OTA and PAT also showed a similar pattern with a slight quadratic effect.
(p = 0.10), but did not exhibit either significant synergism or antagonism at any dilution levels (Figure 7.3B).

The mixture of PAT and MPA showed the strongest antagonistic inhibitory effect on the proliferation of BoMacs among the ten binary mixtures of PMs tested (Figure 7.3H). PAT+MPA showed antagonism across all dilutions (p < 0.05) without the presence of either linear or quadratic relationships. CIT+MPA, CIT+PA and MPA+PA showed significant antagonism at the mixtures of IC25 and 0.5IC25 (p < 0.05; Figure 7.3F, G and J). MPA+PA exhibited a linear relationship between $P_{OM}$ and $P_{EM}$ (p < 0.05). CIT+MPA also showed a slight linear relationship (p = 0.09), while CIT+PA did not.

7.5. Discussion

*Penicillium* mycotoxins (PMs) are common toxic compounds found in stored silage for animal feed (Mansfield et al., 2008), and several studies have demonstrated inhibitory effects of individual PMs on the proliferation of leukocytes (Keblys et al., 2004; Stec et al., 2009). However, the risk observed from the individual PM exposure may not reflect the true risk of PMs due to their potential interactions (Stork et al., 2007), because most PMs are often found in combinations of more than one in animal feed (Mansfield et al., 2008). Previously, we examined the potential interaction among the binary mixtures of PMs, including CIT, OTA, PAT, MPA and PA, at their respective IC25s by using BoMac proliferation (Oh et al., 2012). From the study, we reached a conclusion that the combinations of OTA with CIT, PAT or PA showed synergistic and additive interaction, while the mixture of OTA and MPA showed antagonistic effects. The study, however, was lacking in both experimental and statistical methods; a simple Tukey’s test comparing the percent proliferation of individual PMs to that of PM mixtures may not be sufficient for
determining interactions of PMs. Therefore, we have re-examined the previous data under a proper assumption of IA or CA models.

By using either of two models, the effect of mixtures can be estimated from the effect of individual compounds under the assumption of additivity. After that, the predicted effect can be compared to the observed effect of the mixtures, and therefore, can determine a potential interaction among the compounds by rejecting the assumption of additivity (Kjaerstad et al., 2010; Stork et al., 2007).

Independent action (IA) model assumes that compounds of a mixture have completely independent modes of action affecting the same end point, such as proliferation (Cedergreen et al., 2013; Plackett and Hewlett, 1963). Under the assumption of the IA model, a similar conclusion as the previous paper was reached. For example, OTA in combination with either CIT, OAT or PA had synergistic effects on the proliferation of BoMacs. However, the combinations of PA with either PAT or MPA were determined to have antagonistic effects, but not the mixture of OTA and MPA.

In contrast to the IA model, the CA model assumes that the compounds of a mixture behave as simple dilutions of one another (Cedergreen et al., 2013; Kortenkamp and Altenburger, 1998). While IA uses single substance effect to estimate a mixture effect, the CA model uses effect concentrations to estimate the same effect concentration of the mixture (Cedergreen et al., 2013). In other words, the CA model takes account of the proportion of the concentrations at various effect levels. For example, if the IC25s of CIT and OTA were determined to be 52.71 μM and 8.91 μM (Oh et al., 2012), then IA model takes the ratio of their effect, which is their IC25 in this case; therefore, the ratio between CIT and OTA is 1:1 in IA model. On the contrary, the CA model takes
into account the ratio of concentrations, not the effect, giving a ratio of 6:1 (52.71 μM/8.91 μM) instead of 1:1 from the IA model. Interestingly, under the assumption of the CA model, only OTA+PA showed a synergistic interaction, while PAT+PA was antagonistic to cell proliferation.

So far, the results of the previous proliferation data that were reanalyzed by using the IA or CA model were discussed. Clearly, there are differences in the results between these two models, yet both models seem to be in the agreement that OTA+PA mixture exhibited synergism, while PAT+PA showed antagonism on the proliferation of BoMacs. The synergism of OTA and PA has been also reported in a number of studies. Sansing et al., (1976) described the synergistic toxicity of the mixture on the liver and kidney tissues of mice. Another in vivo feeding study also reported a similar result, in which they observed significant synergistic nephrotoxicity along with the increased mortality rate of mice when these mice consumed both OTA and PA compared to OTA or PA alone (Shepherd et al., 1981). Considering that some common mold species in grain, such as *Penicillium viridicatum* and *Aspergillus ochraceus*, can produce both OTA and PA (Bacon et al., 1973; Sansing et al., 1976), assessing the risk of these two mycotoxins also needs to take into account their potential synergism.

PAT plus PA was another mixture that was identified to have an interaction of antagonism from both models. The interaction between PAT and PA has not been largely studied as compared to that of OTA+PA, but there is one study reporting their potential antagonism. Ashoor and Chu (1973) examined the inhibitory effect of PAT and PA on the in vitro production of yeast alcohol dehydrogenase (ADH) and rabbit-muscle lactic dehydrogenase (LDH). They reported that PAT and PA were non-competitive inhibitors of ADH, but showed a competitive inhibitory effect on LDH production. This suggests that PAT and PA may exhibit antagonism on LDH as they compete with each other to target one specific mode of action to affect LDH production in this case.
However, the study also reported that the mixture also showed an additive effect on ADH production (Ashoor and Chu, 1973). This suggests that the antagonism of PAT and PA may be dependent on different cell types, and this may also apply to other PM mixtures.

The application of IA or CA model provides the threshold point or line of expected additivity effect from the individual exposure data (Cedergreen et al., 2008). This provides a statistical means of distinguishing interactions of synergism, additivity and antagonism between PM mixtures. However, since the mode of action of these PMs on the proliferation is not known, it is unclear which model is suitable to test interactions for the toxicity of PM mixtures. As it was discussed previously, results can differ depending on which model is applied to a study with the same data set available. In the absence of a mode of action, this would depend on which end point a study is focusing on according to the guidelines of the European Commission and Directorate General for Health & Consumers, 2012. For example, if a study focuses on the human or animal health perspective, where it examines an effective level, such as certain signs or symptoms of a compound, then IA model is probably a better model for an initial test. If a study is more interested in looking at the concentrations that cause these signs and symptoms, then CA model is probably the best choice (Cedergreen et al., 2013; European Commission and Directorate General for Health & Consumers, 2012).

When it comes to toxicological testing for mycotoxins, both effect levels and concentrations are important. However, since determining the changes in the effect levels due to the interaction of PMs in mixtures are the primary propose of this study, the IA model is probably a better model to apply for the purpose of this study (Cedergreen et al., 2013). Nevertheless, neither would be any better without the proper information available in determining interactions of mixtures. Previous studies have assessed the observed effect of PM mixtures only at their IC25s (Figure 1 and 2; Oh
et al., 2012). This may describe the interaction or the PM mixtures at their IC25s, but this would not reflect interactions of the mixtures at other concentrations or effect levels due to the lack of experimental data. Therefore, in this study we also tested three IC25 dilutions of PM mixtures to see if their interactions changed over the different effective levels by using the IA model.

The results from the present study showed completely different results as compared to the previous proliferation data. For example, the estimated effect of OTA+PA overlapped with the observed effect of OTA+PA across all dilutions of the mixture, indicating that OTA and PA showed additivity, not synergism, regardless of which dilutions were used. The synergism was rather observed from the mixtures of CIT+OTA and PAT+PA at their 0.5IC25s. The trials were repeated in the same way as the previous proliferation trials except for the use of more treatments and different solvents, where ethanol was used to replace DMSO in this study. It is unclear how exactly the solvent affected the potency and interaction of these PMs, but the solvents, DMSO or ethanol, might have altered the synergistic interactions of these mycotoxins (Crankshaw and Raper, 1971).

The synergism between CIT and OTA was observed at IC25s from previous proliferation data with the IA model. The synergism of CIT+OTA was also observed in the present study, but it did not occur at IC25s but rather occurred at the mixture of 0.5IC25s in the present study. There is no clear reason why the synergism occurred at different dilution levels, but we do know that these two mycotoxins exhibited synergism in inhibiting the proliferation of BoMacs. The synergism between CIT and OTA was also previously reported from other studies. For example, the mixtures of CIT and OTA were previously shown to increase chicken embryonic toxicity compared to CIT or OTA alone (Veselá et al., 1983). Other studies also reported synergistic toxicity of CIT and OTA mixtures on liver and kidney tissues (Sansing et al., 1976; Speijers and Speijers, 2004). Since
the results from the present study indicate that the interaction of CIT and OTA only occurred at the 0.5IC25s, their synergistic toxicity in animals may or may not be observable depending on their biological concentrations in animals.

Similar to the previous proliferation data with the IA or CA model, the present study also determined that the mixture of PAT and PA showed antagonism on the proliferation of BoMacs at IC25s. However, PAT and PA also exhibited a quadratic relationship, where PAT and PA at the combination of 0.5IC25s showed synergistic effects, and additive effects at 0.25IC25s. This suggests that the interaction of PAT and PA is also largely dependent on the concentration, similar to CIT+OTA. The only difference is that PAT+PA showed synergism, antagonism and additive effects depending on their dilution level. Since there is a previous report indicating a possibility that PAT and PA may have the same mode of action for altering the hydrogenase production in yeast and rabbit muscle tissues (Ashoor and Chu, 1973), the CA model would probably provide better information in determining the interaction for this specific mixture.

In comparison to the previous proliferation study, the present study revealed more PM mixtures with antagonistic interactions. The result from this study clearly indicated that PAT and MPA demonstrate antagonism on the proliferation of BoMacs regardless of their dilution levels. Antagonism was also observed from the other PM mixtures, including CIT+MPA, CIT+PA and MPA+PA. This antagonism was largely dependent on the dilution levels, where their significant interaction was most observable at the mixtures of IC25 and 0.5IC25, but not at 0.25IC25. For example, both MPA+PA and CIT+MPA showed significant antagonism with a linear relationship between the observed and estimated proliferation. This indicates that the antagonisms observed from these combinations will likely increase as the concentration of the mixtures increases. For most compounds, interactions, such as antagonism or synergism, generally occur at relatively
medium or high concentrations, but they are not likely to occur at low concentrations (European Commission and Directorate General for Health & Consumers, 2012), and this seems to be the case in most mixtures in this study, where their significant interaction generally diminished as the overall concentration of the compound in the mixtures decreased.

In addition to this, there is a possibility that the compounds in the mixtures that had a linear relationship, including CIT, MPA and PA, may share the same mode of action, targeting the same molecules to inhibit the proliferation of BoMacs when mixed together. The concept is somewhat similar to that of the saturation model used in the kinetics of enzymes (Khera et al., 2014). These mycotoxins at low concentrations would show non-competitive inhibition, and therefore, an additive effect on the proliferation of the BoMacs was observed. When they reach a certain concentration, they compete for the same target rather than going after other targets. Although CIT+PA did not show any linear effect, the mixture still showed significant antagonism at the mixtures of IC25s and 0.5IC25s, similar to that of MPA+PA and CIT+MPA. This is probably what Ashoor and Chu (1973) observed in the mixture of PAT and PA, showing competitive inhibition on ADH production but not on LDH production. The yeast and rabbit tissue may share the similar mode of action in producing dehydrogenases, but the amount of the specific molecules for dehydrogenase production may differ depending on the cell types (Ashoor and Chu, 1973). Since CIT, MPA and PA appear to share the same mode of action for inhibiting the proliferation of BoMacs, the use of CA might have been a better model to test for their interaction (Cedergreen et al., 2008). Interestingly, the combinations of CIT+MPA were also determined to be antagonistic from the IA analysis of the previous proliferation data, but the mixture was determined to have an additive effect when the CA model was applied. Therefore, it is possible that the following
mixtures, MPA+PA, CIT+MPA and CIT+PA, may not show antagonism under the use of the CA model.

7.6. Summary and Conclusion

In the present study, the additivity models were applied to determine the potential interactions among the binary mixtures of PMs. First, the previous proliferation data were reanalyzed by using the IA or CA model. Although the two models came up with different results, both models agreed that the mixture of OTA and PA produced synergism on the proliferation of BoMacs, while the mixture of PAT and PA exhibited antagonism. When the trials were repeated with more concentrations of the binary mixtures by diluting the mixtures of IC25s, CIT+OTA and PAT+PA were shown to have synergism at the combination of their 0.5IC25s. Interestingly, PAT+PA also produced antagonism at the mixture of IC25s as it was previously observed. This indicates that the interaction of PMs was largely dependent on their concentrations in the mixture. Other PM mixtures, including PAT+MPA, CIT+MPA, CIT+PA and MPA+PA, showed the antagonism on the proliferation of BoMacs. However, only PAT+MPA appears to have clear antagonism under the IA model regardless of dilution levels, while the antagonisms of other mixtures was only observable at the combination of IC25s and 0.5IC25s but not at 0.25IC25s. The present study provided an informative initial screening to determine interactions of ten binary PM mixtures. For future study, it would be interesting to apply other complex models, such as ray designs, to establish a full interaction curve for those mixtures that showed significant interaction in this study, such as OTA+CIA, PAT+MPA or PAT+PA (Cedergreen et al., 2013; Meadows et al., 2002; Stork et al., 2007).
Figure 7.1: The comparison between ‘observed’ and ‘estimated’ of mixtures of PMs at their respective IC25s by using the IA model. Clear squares indicate the mean of ‘estimated’ proliferation from the PM mixtures. Black squares represent the mean of ‘observed’ proliferation, and the error-bars are 95% CI. If the mean of estimated proliferation overlaps with 95% CI of observed proliferation, then it indicates there is no significant interaction between two PMs. If the means of estimated proliferation falls above or below the 95% CI of observed proliferation, then it indicates a significant synergism or antagonism, respectively (p < 0.05). (*) denoted significant differences at p-value < 0.05.
Figure 7.2 (A-J): The predicted curve of PM mixtures toxicity by using the CA model.
Figure 7.3 (A-J): The comparison between ‘observed’ and ‘estimated’ of PM mixtures.
Chapter 8: General Discussion and Conclusion

Since the first reported outbreak on a turkey farm in London, England, mycotoxins have generated much attention from the public and researchers as a potential threat, endangering both human and animal food safety (Bennett and Klich, 2003; Forgacs, 1962). Up until now, over 300 biologically active secondary metabolites of filamentous fungi have been identified as mycotoxins (Bennett and Klich, 2003), but relatively few of them, specifically those from *Fusarium* or *Aspergillus* species, have been thoroughly studied regarding their potential toxicity due to their history of outbreak (Forgacs, 1962; Marasas et al., 1987; Ramakrishna et al., 1989). Potential mycotoxicoses of *Penicillium* mycotoxins (PMs), on the other hand, has been widely disregarded despite their high prevalence in crops and grain-based feed for livestock animals, especially ruminant species (Lennox et al., 2003; Mansfield et al., 2008; Pitt, 1987). Therefore, it is important to better understand the risks posed by exposure to these bioactive compounds. To date relatively little is known about the exposure risk to PMs.

A number of PMs, citrinin (CIT), ochratoxin (OTA), patulin (PAT), mycophilic acid (MPA) and penicillic acid (PA) can be found in stored animal feed or food (Lennox et al., 2003; Mansfield et al., 2008). These mycotoxins have been shown to modulate the immune response, which could predispose animals to secondary diseases (Al-Anati and Petzinger, 2006; Ferrante et al., 2008; Herzog-Soares and Freire, 2004). For example, the consumption of these PMs was shown to reduce the number of leukocytes in the blood as well as in primary (thymus and bursa of Fabricus) and secondary lymphoid organs (spleens and lymph nodes) in poultry or rodent species (Chang et al., 1979; Gupta et al., 1979; Reddy et al., 1988; Stoev et al., 2004). More recent *in vitro* studies demonstrated that the PMs decrease leukocyte viability and proliferation, and this likely explains the dramatic reduction of leukocyte numbers reported from *in vivo* studies (Keblys et al., 2004;
Lea et al., 1989; Sorenson et al., 1985; Stec et al., 2008). In agreement with these *in vitro* studies, Chapter 3 of this thesis evaluated the potential effect of the following PMs, including CIT, OTA, PAT, MPA and PA, on cell viability and proliferation of a bovine macrophage cell line (BoMacs) (Oh et al., 2012). These mycotoxins differentially affected the viability of BoMacs; only PAT and PA showed evidence of cytotoxicity, where LC50s (concentration that kills 50% of cells) were determined to be 4.46 μM and 175.79 μM, respectively. The potency of these PMs based on their IC50s (concentration that inhibits 50% cell proliferation) from highest to lowest was: 0.56 μM (PAT) > 12.88 μM (OTA) > 29.85 μM (PA) > 91.20 μM (CIT) > N/A (MPA). The findings in this study support the results from other *in vitro* exposure studies in which the authors listed the same order of potency for these PMs on the proliferation of porcine and bovine lymphocytes (Keblys et al., 2004; Stec et al., 2008). The concentrations determined in the study, such as that of OTA, were higher than circulating concentrations observed from *in vivo* studies (Blank et al., 2003; Höhler et al., 1999). However, there is the potential for their bioaccumulation in certain target tissues, such as kidney, intestine, and cutaneous fat, as well as their interaction with other mycotoxins within these tissues (Sansing et al., 1976).

Besides cell proliferation and viability, some studies have also demonstrated that PMs affect the other functions of macrophages, such as cytokine production, the inflammatory response and phagocytosis (Ferrante et al., 2008; Herzog-Soares and Freire, 2004; Islam et al., 2012; Johannessen et al., 2005; Liu et al., 2003). These functions of macrophages are important for an adequate immune response, and an inappropriate response could potentially increase susceptibility to secondary infection (Ishikawa et al., 1998) and/or lead to auto-immune disease (Cavin et al., 2009). Chapter 5 of this thesis also determined that sub-lethal concentrations of PMs differentially altered the gene expression of inflammatory cytokines in BoMacs. The study also demonstrated
the potential effect of PMs, specifically OTA, PAT and PA, on ROS production and phagocytosis of *Mycobacterium avium* spp. *paratuberculosis* (MAP), an intracellular pathogen that causes Johne’s disease in cattle. In the study, the cytokine gene expression did not necessarily reflect the endpoints measured, such as PAM-3 stimulated ROS production and phagocytosis of MAP. For example, OTA and MPA significantly changed the gene expression of both *IL-1α* and *IL-6*, but these mycotoxins had no effect on ROS production even at higher concentrations. Some cytokines, such as *IL-1α* and *IL-6* are stored in an inactive form in macrophage vacuoles, and become activated and released in response to inflammatory signals, such as LPS stimulation (Maelfait et al., 2008). Therefore, gene expression may not necessarily reflect the actual amount of active cytokine that is released by the macrophage. Given this, future studies may want to explore the effect of PMs on cytokine proteins that are secreted by macrophages.

The study, however, observed some interesting aspects, including that both PAT and PA decreased not only the gene expression of IL-23 but also decreased the intracellular ROS and phagocytosis. A similar phenomenon was also reported from a recent study done by Indramohan and colleagues (2014). These authors observed an equivalent reduction in both ROS production and phagocytosis of *Listeria monocytogenes* in IL-23 knockdown mice as compared to normal C57BL/6 mice (Indramohan et al., 2014). This indicates a possible association among the gene expression of IL-23, intracellular ROS production and phagocytosis of intracellular pathogens. Therefore, it would be interesting to do further correlation studies to determine the relationships among these parameters and to investigate their potential functional interactions in macrophages.

While several studies that have investigated the toxic effect of individual PMs (Keblys et al., 2004; Stec et al., 2008), only a few have assessed the potential interactions among these PMs. PMs in reality do not occur individually, but most often are found in combinations of more than one
mycotoxin (Mansfield et al., 2008; Martins et al., 2002). Furthermore, some of these PMs can exhibit either synergistic or antagonistic interactions, leading to either underestimation or overestimation of the risk associated with mixed compounds (Stork et al., 2007). Chapter 3 of this thesis showed that OTA when combined with CIT, PAT and PA showed potential synergistic inhibitory effects on BoMac proliferation. However, the study was lacking in both experimental and statistical methods for thoroughly investigating PM interactions. Our conclusion was solely based on single concentrations of binary mixtures, and there was no clear line of distinction between additivity, synergism and antagonism.

Therefore, the interactions of binary PM mixtures were re-examined more thoroughly in Chapter 7 under the assumption of additivity using the independent action (IA) and the concentration addition (CA) models. When previous proliferation data were reanalyzed, both the IA and CA models determined synergistic relationships for OTA and PA, and antagonistic relationships for PAT and PA. However, when three different dilutions of IC25 mixtures were subsequently retested using the IA model, the result was completely different from that of the reanalyzed proliferation data from Chapter 3. In Chapter 7, CIT+OTA and PAT+PA showed synergism at medium tested concentrations. The observed proliferation curve of these mixtures also had a quadratic effect in comparison to the estimated proliferation curve. This quadratic effect indicates that the interactions are largely dependent on the effect levels of the mycotoxins in the mixtures. For example, PAT+PA exhibited additivity at low, antagonism at medium and synergism at the high effect level of PM mixtures. The antagonistic effects were also determined in other binary mixtures of PMs, including PAT+MPA, CIT+MPA, CIT+PA and MPA+PA. However, the observed proliferation curve of the following PM mixtures, CIT+MPA and MPA+PA, had a significant linear effect in comparison to the estimated proliferation curve. The linear relationship
between the observed and estimated proliferation curve suggests that the antagonism is dependent on the effect level of PMs, where their antagonistic effects will likely increase as the effect level of the mixtures increase. This study provided an informative initial screening to determine interactions of many binary PM mixtures. However, more complex experimental designs, isobologram and ray ratio designs for example, could be explored in future studies to better approximate PM interactions within mixtures (Meadows et al., 2002; Stork et al., 2007). These models would require much more experimental effort to establish full additivity curves compared to 3-point curves that were used in our study.

In the previous paragraphs, we have summarized that PMs had significant effects on the activities of bovine macrophages (Oh et al., 2012; Sorenson et al., 1985, 1986). These macrophage functions are under the control of epigenetic regulation (Bowdridge and Gause, 2010; Ishii et al., 2009). Therefore, there is a possibility that the effect of PMs observed may be attributed to the changes in epigenetic patterns, such as DNA or histone acetylation or methylation patterns, and the activities of the enzymes regulating them (Marin-Kuan et al., 2007, 2008). Chapter 4 of this thesis demonstrated that individual PMs at their respective IC25s did not affect the gene expression of epigenetic enzymes. However, when OTA was co-exposed with either CIT or PA, a strong synergistic effect on the gene expression of the epigenetic enzymes methyltransferases (DNMT)-3s, histone demethylase (JMJD-3) and histone deacetylase (HDAC)-3 was observed. Studies have suggested that the activities of these enzymes are closely linked to cell proliferation, viability, and inflammatory response (Begriche et al., 2011; Kurita et al., 2010; Sun et al., 2011; Wilson et al., 2006). Therefore, it is possible that the synergistic suppression in BoMacs’ proliferation that was observed in Chapter 3 might be due to the changes in the expression of the epigenetic enzymes observed in Chapter 4 of this thesis. Unfortunately due to time constraints, we did not evaluate the
potential synergistic effect of these combinations on the gene expression of cytokines, ROS production and phagocytosis. Therefore, it will be important to address the potential synergistic effect of PMs on these parameters to determine the possible link between the epigenetic changes and the macrophage functions.

So far, it was discussed in this thesis that PMs have potential toxic effects on BoMacs, specifically, that these mycotoxins affected the cell proliferation, viability, cytokine gene expression, ROS production and phagocytosis of MAP with respect to the bovine macrophages (Oh et al., 2012, 2013). PMs differentially modulated the macrophage functions and viability, and this in turn, could predispose animals to secondary diseases, such as Johne’s disease. The next important step after determining their potential immunomodulatory effect would be to find possible solutions to resolve these issues. Therefore, Chapter 6 of this thesis looked into the efficacy of MA+ for preventing the potential toxicity of PMs using BoMacs proliferation as a bioassay endpoint. From this study, MA+ showed the highest efficacy preventing OTA toxicity at pH 3.0 but not at pH 5 or 7. Raju and Devegowda, (2002, 2000) also demonstrated a beneficial effect of MA+ for preventing OTA toxicity using both an in vitro binding test and in vivo studies with broiler chicks. MA+ also showed a preventive effect on CIT toxicity, but this required a longer incubation time than 6 h and a higher percent inclusion of MA+ than 0.5%. MA+ was not effective for preventing the toxicity of the other mycotoxins, PAT, MPA and PA, tested in the trial. There are, however, other factors to consider, such as temperature and the mineral content in the incubation media. These factors could also largely contribute to the efficacy of MA+ as well as the toxic potency of mycotoxins (Evans and Dawson, 2000; Fruhauf et al., 2012).

Overall, this study provides insight into the potential immunomodulatory effect of PMs by using the BoMacs in vitro model. The study demonstrated that PMs differentially affected cell
viability, proliferation, ROS production, phagocytosis as well as the gene expression of the cytokines and epigenetic enzymes that regulate cellular activities. However, all the trials were carried out as *in vitro* studies using a bovine macrophage cell line (BoMacs). In comparison to *in vivo* studies, *in vitro* studies provide more control of variables, and allow researchers to investigate specific effects of mycotoxins (Emami, 2006). At the same time, it is a reality that most *in vitro* studies, including the trials in this thesis, do not consider all other biological and environmental factors that could alter the effect of mycotoxins or MA+ efficacy (Emami, 2006; Raju and Devegowda, 2002, 2000). Therefore, it would also be important to continue on with some follow-up studies to examine the immunomodulation of PMs by using *in vivo* ruminant models in relation to the prevalence of common infectious diseases, such as Johne’s disease.

In the end, the most important question to ask would be, “Can these *Penicillium* mycotoxicoses be prevented?” in terms of the animal industry’s view. Proper feed management is the most effective way to reduce the risk of mycotoxin occurrence in livestock feed post-harvest. However, if a high prevalence of mycotoxins is observed, there are several methods, such as inclusion of clay or other mycotoxin binders, such as GMA or MA+, available to reduce the risk of mycotoxicoses in animals (reviewed by Kabak et al. 2006). This, however, would depend on the types of PMs, as the efficacy of mycotoxin binders largely varies with different PMs (Fruhauf et al., 2012; Kabak et al., 2006). In addition to this, in reality, mycotoxins do not occur individually but most often are found in combinations in ensiled grain- and/or crop-based livestock feed, making it difficult to truly assess their risk and toxicity (Mansfield et al., 2008; Oh et al., 2012; Stoev et al., 2004; Veselá et al., 1983). Therefore, in order to gain a greater perspective on how to detect and prevent mycotoxins in the agricultural industry, combinational mycotoxicoses should be considered in future studies.
References


Appendix 1: Schematic diagram of how Penicillium mycotoxins alter the cell activities.

Green arrow represents ‘stimulation’, and red indication represents ‘inhibition.’ MPA or PA inhibits HDAC-3 expression and Bmi-1 increasing p21 activity, which then down-regulate the proliferation (Spurling et al., 2008; Wilson et al., 2006; Wong et al., 2011). The halted proliferation by p21 can also stimulate the differentiation of the cells. PA stimulates JMJD-3 expression, which can also induce p21 activity by inducing p53 (Barradas et al., 2009; Bringold and Serrano, 2000). Although PA inhibits DNMT-3b, it does not alter the p19/ARF and p16/INK4A activities as DNMT-1 is still active (Kurita et al., 2010).
Appendix 2: Preliminary study on the effect of 6 h in vitro PM exposure at different concentrations on BoMac ROS production.

‘Blue rectangle’ with blue line indicates the % ROS production in relative to control; ‘black dotted line’ represents the cell viability. ‘Red arrow’ indicates IC25 for each PMs. Prior to selecting the exposure concentrations for ROS production (Table 5.1), wide ranges of PM concentrations were tested to determine approximate concentration range that may have altered the ROS production.
Appendix 3: Intracellular ROS production of BoMacs with different stimulators by using Carboxy-H2DFFDA.

Only PAM-3 at 10 μg/ml dramatically induced the ROS production in BoMacs after 3 h of incubation. 24 h of PAM-3 had higher intracellular ROS reading as compared to 6 h of PAM-3. However, the most increase was observed in between 3 to 6 h of incubation, so 6 h incubation with 10 μg/ml PAM-3 was used as optimized ROS assay condition with H2DFFDA dye.
Appendix 4: Effect of PAT on the viability of BoMacs.

To determine if PAT incubation with MA+ decrease the cell viability. (See section 6.3 for more detail method). Prior to freezing down the plates for proliferation assay, ethidium homodimer-1 (excitation 528/emission 617 nm) were used to estimate the death cell number from the half of the replicates (triplicates) of treatments. The cell number from proliferation assay indicated the total cell number from the treatment, and the measurement from Eth-1 solution indicated the dead cell number. The percent in the graph was estimated by dividing dead cell number from the total cell number. (This step was necessary, since the total cell number was affected depending on the percent amount of MA+ as it was shown in Figure 3C. The study showed that the cell death increased as the percent MA+ increased.
Appendix 5: Effect of MA+ on BoMac proliferation.

The respective percent of MA+ was mixed with water at pH 7.0 and incubated for 2 h. The MA+ was then removed by centrifugation at 10,000 g for 10 min before the cell culture procedure. Calcein AM (excitation 494/emission 517 nm) was used to measure the cell viability (see section 3.3.2. in Chapter 3 for detail).