The *in vitro* Modulation of Intestinal Growth and Inflammatory Indices by Fumonisin B_1 and Hydrolyzed Fumonisin B_1 through Protein Network Analyses

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DECLARATION

I, **Nabeela Gamiet**, declare that the contents of this thesis are an original representation of my own work, has been composed by me, and has not previously been submitted for any academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed: N.Gamiet **Date:** **05 March 2024**

ABSTRACT

There is an increasing amount of staple feed and food in low- and middle-income countries that can be contaminated by various mycotoxins. Regarding this, tthe intestinal epithelium is constantly being exposed to these ingested harmful contaminants, such as mycotoxins. Fumonisin B_1 (FB_1) is one of the most toxic and abundant fumonisins found in nature and commonly contaminates maize and maize-based feed and food, causing various adverse effects in many mammalian species. This can often lead to various forms of infection and inflammation, which trigger stress response pathways, promoting delamination of cells and inducing apoptosis. FB_1 can be enzymatically converted to form a less potent ceramide synthase inhibitor, hydrolyzed fumonisin B_1 (HFB_1), which was found to have controversial results in various *in vitro* and *in vivo* models. Many studies have focused on *in vivo* FB_1 and HFB_1 individually, but have failed to identify the *in vitro* comparative mechanisms regulating cellular interactions during fumonisin exposure. The identification of various signaling pathways provided after protein analysis, contributes to the increased understanding of the mechanisms behind such pathogenic interactions. In the present study, the modulating effect of FB_1, HFB_1 and lipopolysaccharides (LPS) on the growth and immune indices of the intestinal porcine enterocyte (IPEC-J2) cell line was investigated. It was then followed by the identification of molecular mechanisms and pathways influenced by FB_1 and HFB_1, and a comparative proteomic analysis was performed. Initially, IPEC-J2 cells were exposed to various concentrations of FB_1, HFB_1 and LPS. Thereafter the modulating effect of FB_1 and HFB_1 on the growth and immune indices was investigated. The cell survival indices (cell viability, apoptosis, and cell proliferation) were measured, while inflammatory responses were monitored by immune-detection of interleukin 8 (IL-8). Co-exposure of FB_1 and HFB_1 with LPS were also analyzed. It was found that HFB_1 elicits a heightened degree of toxicity on intestinal epithelial cells *in vitro* compared to FB_1. IPEC-J2 cells were then exposed to 7.81 µM and 15.63 µM concentrations of both FB_1 and HFB_1 for 24 hours, respectively. Cells were quantified through proteomic analyses using liquid chromatography- mass spectrometry (LC-MS/ MS). Bioinformatics analyses were conducted, and Differentially Abundant Proteins (DAPs) were identified and visualized with volcano plots. The functional annotation of DAPs was carried out using Gene Ontology (GO), comparing data using the *Homo sapiens* and porcine databases. A total of 52 significant DAPs were identified between FB_1 and HFB_1 compared to the control. In the KEGG pathways enrichment analysis, 15.63 µM FB_1 exposure elicited a significant enrichment of proteins within multiple cancer pathways and the AGE/ RAGE signaling pathway. During 7.81 µM HFB_1 exposure, a significant enrichment of proteins was identified within ribosomal pathways, while exposure to 15.63 µM HFB_1 elicited a

significant enrichment of proteins within the ECM receptor interaction and proteoglycans in cancer, as well as focal adhesion, and bacterial invasion of epithelial cells. Fibronectin 1 (FN1), an adhesive glycoprotein of the intestine, was the only protein observed amongst all concentrations in cells exposed to FB_1 and HFB_1. It was found that FB_1 up-regulates FN1 and HFB_1 down-regulates FN1, which in turn elicited very different cancer promoting pathways using Cytoscape and STRING enrichment analysis. The expression of FN1 is important in cellular integrity maintenance, response to intestinal epithelial injury, and wound healing. Individually, it is noted that HFB_1 promotes a greater toxicity on intestinal epithelial cells *in vitro* compared to FB_1. Although HFB_1 is known to be less toxic compared to FB_1 *in vivo*, which suggests that HFB_1 is metabolized differently *in vitro* compared to *in vivo*. This finding was suggested based on the individual and co-exposure data obtained. The potency of FB toxicity on intestinal cells are affected by the complexity of pathways connected. These results further suggest that HFB_1 promotes a greater toxicity upon the IPEC-J2 cell line when compared to FB_1, due to the abundance of proteins that were affected during exposure and the interconnectedness of pathways that were enriched.

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DEDICATION

I would like to dedicate this thesis to my husband and daughter. You are the pinnacle of my motivation, strength and success. The best words to describe our experiences throughout this period would be:

“And we will provide for him from where he does not expect.”
-Quraan (65:3)

TABLE OF CONTENTS

ABBREVIATIONS

ACN	Acetonitrile
ACTN2	Actinin alpha 2
AF	Aflatoxin
AMHBI	Applied Microbial and Health Biotechnology Institute
ANOVA	Analysis of variance
Anti-BrdU-POD	Anti-5'bromo-2'-deoxyuridine-peroxidase
AP1	Aminopentol
ARFGEF1	ADP ribosylation factor guanine nucleoside
ATF-3	AMP-dependent transcription factor
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BH adjusted	Banjamini-Hochberg adjusted
BP	Biological processes
BrdU	5'bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CC	Cellular components
CCN3	Cellular communication
CDH6	Cadherin 6
CDK6	Cyclin-dependent kinase 6
CerS	Ceramide synthase
CHCHD2	Coiled-coiled helix coiled-coiled helix domain-containing 2
CO_2	Carbon dioxide
CoA	Coenzyme A
COL1A1	Collagen type 1, alpha 1 chain
CPGR	Centre of Proteomic and Genomic Research

CSH	Charged surface hybrid
DAPs	Differentially abundant proteins
DMEM HAMS F-12	Dulbecco’s Modified Eagles medium-Ham’s F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DPBS	Dulbecco’s Phosphate Buffered Saline
DTT	Dithiothreitol
EA	Ergot alkaloids
ECM	Extracellular matrix
EE	Environmental enteropathy
EGF	epidermal growth factor
ELEM	Leukoencephalomacia
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FA	Fatty acid
FB	Fumonisins
FB_1	Fumonisin B1
FBLN2	Fibulin 2
FBS	Fetal bovine serum
FC	Fold change
FDR	False discovery rate
FN1	Fibronectin 1
FTH1	Ferritin heavy chain
FTL	Ferritin light chain
GIT	Gastrointestinal tract

GO	Gene Ontology
Gu-HCL	Guanidine hydrochloride
HBSS	Hank’s buffered salt solution
HCC	hepatocellular carcinoma
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HFB_1	Hydrolysed fumonisin B1
HILIC	Hydrophilic interaction liquid chromatography
IAA	Iodoacetamide
IEC	Intestinal epithelial cells
IFNγ	Interferon γ
IL-1	Interleukin-1
IL-17	Interleukin-17
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IPEC-J2	Intestinal porcine enterocytes
ITAGA/ B	Integrin subunit alpha/ beta
ITM2B	Integral membrane protein 2B
ITS	Insulin-transferrin-selenite
KEGG	Kyoto encyclopedia of genes and genomes
LAMC1	Laminin subunit gamma-1
LC- MS/ MS	Liquid chromatography- mass spectrometry with tandem mass spectrometry
LC water	Demineralized water
LC-MS	Liquid chromatography- mass spectrometry
LN_2	Liquid nitrogen

LPS	Lipopolysaccharides
MALDI	Matrix-assisted laser desorption ionization
MeOH	Methanol
MF	Molecular functions
MOMP	Mitochondrial outer membrane permeabilization
MPs	Mictrotiter plates
mRNA	messenger ribonucleic acid
MS	Mass spectrometry
MTBE	Tert-butyl methyl ether
NTDs	Neural tube defects
NTN4	Netrin 4
OGP	Octyl β-D-glucopyranoside
OTA	Orchratoxin
PAMPs	Pathogen association molecular patterns
Pen/Strep/Amph	Penicillin-Streptomycin-Amphotericin B
PPI	Protein-protein interaction
RBP4	retinol binding protein 4
RLU	Relative light units
ROS	Reactive oxygen species
Sa	Sphinganine
SDC	Syndecan
SDS	Sodium dodecyl sulphate
SIMPLEX	Simultaneous metabolite protein, lipid extraction
So	Sphingosine
SPARC	Secreted protein acidic and cysteine rich
ST14	Suppressor of tumourgenicity 14 protein

STRING	Search tool for the retrieval of interacting genes/ proteins
TEAB	Triethylammonium bicarbonate buffer
TFRC	Transferrin receptor 1
TGFBR2	TGF- β receptor type 2
TGF-β	Transforming growth factor-β
TINAGL1	Tubulointerstitial nephritis antigen like 1 protein
TNF-α	Tumour necrosis factor α
UPLC	Ultra- high performance liquid chromatography
ZEA	Zearalenone

CLARIFICATION OF TERMS

Apoptosis: Apoptosis is defined as programmable cell death found within higher eukaryotic cells and represents a process involved in cellular development and differentiation.

Bioinformatics: Bioinformatics combines biology, computer science, information engineering, mathematics and statistics to analyse and interpret biological data.

Cell proliferation: Cell number increase due to sufficient cell growth and division

Cell viability: A viability assay is an assay to determine the ability of organs, cells or tissues to maintain or recover viability.

Contaminant: A polluting or poisonous substance that makes something impure

Environmental enteropathy: Environmental enteropathy (EE) is a subclinical condition, presented as villous atrophy, crypt hyperplasia, and modest malabsorption.

Fumonisin: Fumonisins are secondary metabolites produced by fungi growing mainly on maize.

Inflammation: A process by which the immune system releases immune cells and mediators to defend the body against harmful pathogens

Intestinal epithelium: A monolayer of tightly packed intestinal cells, with important roles in the cellular absorption, transport, along with immunity and barrier integrity functions.

IPEC-J2: Intestinal porcine enterocyte, isolated from the jejunal epithelium of neonatal un-suckled piglets.

LC-MS/ MS: Liquid Chromatography Tandem Mass Spectrometry is a technique used to fragment specific peptides that can be used to obtain an amino acid sequence.

Mycotoxins: Naturally occurring toxins produced by various fungal species, commonly infecting certain plants, feeds and foods.

Proteins: A complex substance joined by peptide bonds and consisting of amino acids residues.

Proteomics analysis: The identification and quantification of proteins within a particular cell, tissue, organ, or organism at a specific point in time

Stunting: A particular animal or human who is too short for their age due to recurrent malnutrition and pathogen exposure

LIST OF FIGURES

LIST OF TABLES

APPENDICES

PREFACE

This thesis is written in an article-based format and consists of 5 chapters. Chapter 1 includes a general introduction, aim and objectives, research statement and rationale. Chapter 2 is the literature review that provides a detailed description of mycotoxins, fumonisin, the gut and barrier function, as well as proteomics. Chapter 3 focuses on the IPEC-J2 cell line model development with FB_1, HFB_1 and LPS individually and within a co-exposure model. Chapter 4 continues with the protein analysis of the IPEC-J2 cell line after FB_1 and HFB_1 individual as well as co-exposure though protein identification and pathway associations. Chapter 5 concludes the thesis with a summary of the discussions and conclusions of the entire thesis, while also mentioning the limitations undergone and applications for future research. Chapters 3 and 4 consist of separate abstracts, introductions, materials and methods, results and discussions as it focuses on different aspects of the thesis.

CHAPTER 1: Introduction

1.1. Introduction

Children within low- and middle-income countries are prone to increasing adverse effects to intestinal health through the ingestion of harmful contaminants (Smith *et al.*, 2012). Maize and maize-based food products are frequently being exposed to contaminants within these countries due to poor pre- and post-harvest agricultural practices (Geary *et al.*, 2016). Mycotoxins such as fumonisin are commonly contaminating these foods, which is often associated with adverse effects upon the infected crop and subsequently, animals and humans ingesting it (Bennett and Klich, 2003).

The intestinal epithelium is the first line of host defence and any dysfunction leads to a variety of adverse effects (Szabó *et al.*, 2023). The tightly packed columnar epithelial cells play an important role in the absorptive and digestive maintenance, alongside barrier and immune functions (Subramanian *et al.*, 2020). Therefore, maintenance of optimal intestinal function is crucial for development and the prevention of pathogen invasion, as it leads to intestinal dysfunction through induction of inflammation and apoptosis (Apidianakis *et al.*, 2009). Intestinal dysfunction is an observable link between pathogen invasion and immune response. The presence of cytokines contributes to optimal immune function and are regulators and mediators of inflammation.

Fumonisin B_1 (FB_1) is one of the most toxic and abundant secondary metabolite mycotoxins found in nature (Aydinoglu, 2021). FB_1 inhibits ceramide synthase and causes multiple adverse effects on various mammalian species (Bertero *et al.*, 2018). Growth impairment through intestinal dysfunctional is one of many adverse effects potentially caused by FB_1 exposure (Bouhet and Oswald, 2007, Loiseau *et al.*, 2007). Additionally, it was observed that the pig is the most susceptible species to FB_1, causing severe adverse effects on the lung, heart and liver (Devriendt *et al.*, 2009, Haschek *et al.*, 2001). FB_1 can also be microbially degraded to a less toxic metabolite, hydrolyzed fumonisin B_1 (HFB_1), which is known to be pro-inflammatory and induce cancer, but to a lesser extent to that of FB_1 (Gu *et al.*, 2019, Humpf *et al.*, 1998). However, it has been observed that HFB_1 presents controversial results under certain conditions, which is poorly understood (Caloni *et al.*, 2005, Dellafiora *et al.*, 2018, Hartl and Humpf, 2000). Further investigation on the mechanisms behind the induction of toxicity has to performed and analyzed.

The inclusion of proteomics within this study aids to the prior information obtained about the effect of FB_1 and HFB_1 on porcine intestinal cells. Proteomics provide greater knowledge on the proteins responsible for regulating key cellular processes, through the identification of protein structure, pathway, expression and function (Pierce *et al.*, 2007). Identifying the biological functions from protein synthesis and functioning, also relates to an increased

understanding of the underlying mechanisms related to disease (Yaffe, 2019). It is suggested that proteins are the predictors of disease progression due to the control and regulation of majority of the biological processes (Macklin *et al.*, 2020, Yaffe, 2019). The identification of protein characteristics, provide increased information about how certain diseases are induced and which pathways could be the driving force of disease progression. Therefore, understanding and subsequently working on ways to prevent disease in the future.

The toxicity of FB_1 and HFB_1 have been evaluated by many previous studies. However, the mechanism behind the toxicity and comparative interaction with intestinal epithelium is poorly understood. Therefore, the mechanism behind the interaction of FB_1 and HFB_1 had to be further evaluated. In the current study, porcine intestinal cells were exposed to various concentrations of FB_1, HFB_1 and LPS to determine the toxic effects upon the cell line via the identification of various cell survival indices alongside inflammatory markers. The interaction was further investigated via the identification of proteins that are affected by FB_1 and HFB_1 exposure, through proteomics analyses.

1.2. Aim of the study:

To elucidate the mechanisms of fumonisin B_1 and hydrolyzed fumonisin B_1-induced modulation of gut integrity and immune response function, while utilizing omics technology.

1.3. Objectives of the study:

- To maintain and perform routine procedures on cell culture of the intestinal porcine enterocytes, IPEC-J2.
- To establish the optimal exposure conditions with FB_1, HFB_1 dose, and LPS regarding the modulation cell survival indices (apoptosis, cell proliferation, cell viability).
- To determine the effect of FB_1, HFB_1, and LPS on inflammatory parameters using a porcine IL-8 ELISA.
- To establish the optimal co-exposure conditions regarding FB_1 and HFB_1 dose with LPS.
- To investigate the effect of FB_1 and HFB_1 on important protein parameters of intestinal epithelial cells through proteomics and bioinformatics analyses.

1.4. Research statement

The modulation of intestinal cell integrity and inflammatory responses by fumonisin B_1 and hydrolyzed fumonisin B_1 in pig cells *in vitro*: Development of a proteomic interactive cell model.

1.5. Research rationale

Childhood stunting is an important and intractable public health problem that underlies 20 % of deaths among children < 5 years in developing countries. Environmental enteropathy (EE), a subclinical condition of the small intestine characterized by reduced absorptive capacity, and increased intestinal permeability, is common among children in developing countries and may contribute to stunting. However, the etiology of EE is poorly understood. In this regard mycotoxins frequently contaminate the staple foods of populations living in developing countries. Although these toxins have distinct actions, they mediate intestinal damage through i) inhibition of protein synthesis (aflatoxin and deoxynivalenol) ii) an increase in systemic pro-inflammatory cytokines (deoxynivalenol) and iii) inhibition of lipid biosynthesis (fumonisin).

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CHAPTER 2: Literature Review

2.1 Introduction

Children in developing countries are more susceptible to the exposure of harmful agents which adversely affect their growth and development (Chen *et al.*, 2018). These issues are often followed by significant damage to intestinal health. An emerging concern is frequent exposure to mycotoxins that contaminates a wide range of staple foods within these countries.

2.2 Mycotoxins

Mycotoxins are low molecular weight, secondary metabolites that are produced by various filamentous fungi, contaminating feed and food (Bennett and Klich, 2003, Bouhet *et al.*, 2004). The term “mycotoxin” became prominent after 1962, when a veterinary crisis occurred near London, England. The discovery of the causal agent, aflatoxin contamination, for Turkey X disease was the turning point and escalation for mycotoxin research (Bennett and Klich, 2003, Blount, 1961). In this regard, mycotoxins commonly contaminate feed and food products due to infestation of crops related to pre- and post-harvest agricultural practices (Yiannikouris and Jouany, 2002). The effect of mycotoxins on humans and animals mainly depends on the level of contamination, exposure period, type of toxin, nutritional status, and additional synergetic or additive effects with other chemicals. Whereas, the growth and production of mycotoxins from fungi are affected by many factors such as temperature, pH, humidity, water activity, nutrients and microbial interactions (Garcia *et al.*, 2009, in't Veld, 1996). It is quite challenging to both define and classify mycotoxins independently due its broad but interactive biological and chemical capacity (Bennett, 1987). It was found that many mycotoxins may exhibit multiple or overlying toxicities among vertebrates, plant species and other microorganisms. Additionally, multiple mycotoxins can infect a single substrate (Zain, 2011). Mycotoxins are made from diverse chemical structures with various biosynthetic origins. Therefore, classification is usually reflected by the qualification and educational background of the person categorizing it. For example, clinicians would arrange them by the organ it affects, cell biologists arrange them via generic groups, organic chemists classify them by comparing similar chemical structures, and mycologists by the fungi that are produced (Bennett and Klich, 2003). The effect of mycotoxins on animal and human health is known as mycotoxicosis. It is commonly obtained via ingestion of plant-derived foods contaminated with mycotoxins but can also be obtained via dermal contact or inhalation of spores (Bennett, 1987, CAST, 2003, Meyer *et al.*, 2003, Zain, 2011). Primary mycotoxicosis occurs when the mycotoxin-infected plant is consumed directly by the human or animal, presenting adverse effects; and secondary mycotoxicosis occurs when the infected plant is consumed by an animal and its meat or milk products (where the mycotoxin binds in the tissues and is secreted in the milk) are consumed by humans (Smith and Moss, 1985). Mycotoxicosis is commonly categorized into acute and

chronic toxicity (Bennett and Klich, 2003). Acute toxicity is a rapid adverse effect, occurring within a relatively short interval following exposure of the toxin. Chronic toxicity is a permanent or long-lasting adverse effect occurring after a long period of exposure to the toxin (Roberts *et al.*, 2015). The effect of mycotoxins on various animal and human organ systems are broad and can be categorized into having carcinogenic, mutagenic, teratogenic, estrogenic, hemorrhagic, immunotoxic, nephrotoxic, hepatotoxic, dermotoxic, neurotoxic properties as well as having adverse effects on the hematopoietic system (CAST, 2003, Milićević *et al.*, 2010).

Presently, approximately 400 mycotoxins have been identified, with most derived as strains from major mycotoxin-producing species, such as *Aspergillus* producing aflatoxin (AF), *Claviceps* producing ergot alkaloids*, Fusarium* producing fumonisins (FB), zearalenone (ZEA), and deoxynivalenol (DON), and *Penicillium* producing orchratoxin (OTA) (Agriopoulou *et al.*, 2020). These major mycotoxin-producing species together with its metabolites and overall effects on humans and animals are presented in Table 1. However, depending on the target and concentration of the metabolite, not all toxic compounds produced by fungal species are harmful mycotoxins. For example, *Penicillium chrysogenum* is toxic to bacteria and is commonly used as an antibiotic (penicillin) in Western medicine. Other fungal species such as fumonisins, are phytotoxic (toxic to plants) but do not affect plant pathogenesis (Bennett and Klich, 2003). These multi-functional properties provide additional complexities as to how mycotoxins could affect animals and humans at various levels of toxicity.

Table 1 : An outline of various mycotoxins produced by major mycotoxin-producing species

Type of mycotoxin	Fungal species	Mycotoxin subgroups	IARC Classification	Effect on humans and animals	References
Aflatoxin (AF)	*Aspergillus flavus, A. paraciticus*	AFB_1, AFB_2, AFG_1, AFG_2 AFM_1, AFM_2	Group 1 carcinogen Group 2B carcinogen	Impaired growth, immunosuppressor, teratogenic, genotoxic, and hepatotoxic (Liver cancer and acute hepatitis) in humans and animals	IARC (2002) Gong *et al.* (2016) Ismail *et al.* (2021) Schroeder and Boller (1973)
Ochratoxin (OTA)	*Aspergillus ochraceus, A. carbonarius, A. niger, Penicillium verrucosum*	OTA, OTB, OTC, OTα, OTβ	Group 2B carcinogen	Nephrotoxic (Possible cause for Endemic Balkan Nephropathy in humans), immunosuppressor, genotoxic and teratogenic, and possibly carcinogenic in animals	IARC (2002) El Khoury and Atoui (2010) Pitt *et al.* (2000) Marín *et al.* (2018) Damiano *et al.* (2018)
Ergot Alkaloids (EA)	*Claviceps purpurea* *C. fusiformis* *C. Africana*	Ergometrine, Ergocornine, Ergosine, Ergokryptine, Ergotamine, Ergocristine	unclassified	Ergotism (St Anthony's Fire) in humans and animals	Agriopoulou (2021) Agriopoulou *et al.* (2020) Flieger *et al.* (1997)
Fumonisin (FB)	*Fusarium verticillioides, F. proliferatum, Alternaria alternata* (A total of 15 Fusarium species are fumonisin producers)	FB series: FB_1, FB_2, FB_3 (28 analogs- A, C, P and Pγ series)	Group 2B carcinogen	Porcine pulmonary edema, equine leukoencephalomalacia, hepatotoxic in pigs, horses, cattle, rabbits and primates. Hepatocarcinogenic in rats and mice, Nephrotoxic in rats, rabbits, and sheep, In humans there is a possible association with: Stunting, neural tube defects, and oesophageal cancer	IARC (2002) Rheeder *et al.* (2002b) Rheeder *et al.* (1992) Colvin *et al.* (1993) Marasas *et al.* (1976) Tola and Kebede (2016) Kimanya *et al.* (2010) Gelineau-van Waes *et al.* (2009)
Deoxynivalenol (DON)	*Fusarium graminearum, F. culmorum*	Member of the type B trichothecenes family	Group 3	**Acute and chronic toxicity in animals:** (pigs being the most sensitive species) Emesis (acute), anorexia, growth retardation, immunotoxicity, impaired maternal reproduction and development (chronic). **Toxicity in humans:** Gasteroenteritis, impared growth, neuroendocrine function and immunity	Miller *et al.* (1991) Pestka and Smolinski (2005) IARC and WHO (1993) Sobrova *et al.* (2010)
Zearalenone (ZEA)	*Fusarium graminearum, F. culmorum, F. cerealis, F. equiseti, F. crookwellense, F. semitectum*	α-ZEA, β-ZEA, α -ZAL, β-ZAL, ZON	Group 3	Hyperestrogenism in laboratory and domestic animals (prepubertal swine being the most sensitive) Acts as a naturally-occuring estrogen in humans and promote endocrine disrupting effects	IARC and WHO (1993) Tola and Kebede (2016) Rai *et al.* (2020) Bottalico *et al.* (1985) Kuiper-Goodman *et al.* (1987) (Kowalska *et al.*, 2016)

2.3 Fumonisins

2.3.1. Fumonisin occurrence

Fumonisins (FB) are mycotoxins identified in 1988, and isolated from various *Fusarium* species, predominantly from *Fusarium verticillioides* (formally *F. moniliforme*) (Bezuidenhout *et al.*, 1988, Gelderblom *et al.*, 1988). Other common fumonisin producing species are *F. proliferatum, F. anthophilum, F. dlamini, F. napiforme* and *F. nygamai (Nelson et al., 1992).* Presently, according to Rheeder *et al.* (2002a) at least 28 analogs have been identified and are grouped into various series, namely: A, B, C and P. Fumonisin B_1 (FB_1), (Figure 1) is the most abundant and toxic, amounting to approximately 70 – 80 % of the total naturally occurring fumonisins produced (Rheeder *et al.*, 2002a). Moreover FB is a ubiquitous mycotoxin and classified as a group 2B carcinogen (possibly carcinogenic to humans) (IARC, 2002), which commonly contaminates maize and maize-based products (Rheeder *et al.*, 2002a). Contamination by FB is subject to agroclimatic conditions, insect injury, and plant characteristics which thrives in tropical and sub-tropical climates (Kamle *et al.*, 2019, Wu *et al.*, 2011). Warmer climates, increased rainfall patterns and longer periods of drought provide the optimal conditions for sporulation and germination of *Fusarium* species and subsequent fumonisin contamination (Ahangarkani *et al.*, 2014).

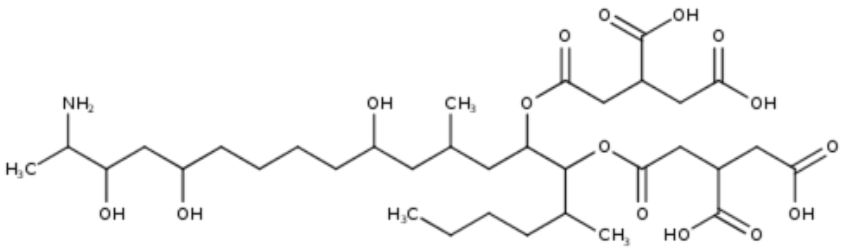

Figure 1: The chemical structure of Fumonisin B_1 taken from the *Toxin and Toxin Target Database* (http://www.t3db.ca/toxins/T3D3603)

2.3.2. Fumonisin B_1 toxicity and interactions

Fumonisin B_1 (FB_1) is structurally similar to the long-chain backbones of sphingoid bases, sphinganine (Sa) and sphingosine (So). It disrupts sphingolipid, lipid, and fatty acid (FA) metabolism by inhibiting ceramide synthase (CerS), an enzyme that acylates sphingoid bases in the synthesis and breakdown of sphingolipids (Iessi *et al.*, 2020, Turner *et al.*, 1999, Wang *et al.*, 1991). During CerS inhibition, sphingoid bases (Sa, So, and 1-deoxysphinganine) together with Sphingosine-1-phosphate and sphinganine-1-phosphate become elevated; while complex sphingolipids, such as ceramide, dihydroceramide, 1-deoxydihidroceramide and associated sphingolipids (sphingomyelin and glycosphingolipids) are reduced (Merrill Jr *et al.*, 1993, Riley and Merrill, 2019a). It was found that FB_1 obstructs the incorporation of [^{14}C]serine into the sphingosine backbone of cellular sphingolipids of rat hepatocytes, as well as a renal cell line (Wang *et al.*, 1991, Yoo *et al.*, 1992). An increase of free Sa was also detected in tissues and serum of fumonisin fed animals (Riley *et al.*, 1993, Wang *et al.*, 1992). Additionally, Merrill Jr *et al.* (1993) found that FB_1 acts in a competitive manner with Sa and stearoyl-CoA to inhibit CerS in mouse brain microsomes, thereby inhibiting sphingolipid biosynthesis and causing a reduction of complex sphingolipids *in situ*. It appears that FB_1 interacts mostly with the binding sites of Sa and fatty acyl-CoA as the potency of inhibition is often associated with the concentration of both substrates (Merrill Jr *et al.*, 2001). It is evident that the biological and physiological target site of FB_1 have been established, providing a relevant pathway for previously hypothesized routes (Riley and Merrill, 2019b).

The effect of FB_1 on various lipid constituents have also been investigated both *in vitro* and *in vivo* which produced a distinct 'lipogenic phenotype' (Gelderblom *et al.*, 1996a, Gelderblom *et al.*, 1997, Riedel *et al.*, 2015). The lipogenic phenotype is the activated genetic program of the *de novo* FA synthesis, including lipogenic enzymes and metabolic regulators, which are linked to the glycolytic metabolism of various cancer progression components (Menendez and Lupu, 2007). The distinct effect of FB_1 on lipid metabolism was observed by a reduction in sphingomyelin, an increase in cholesterol and phosphatidylethanolamine, as well as the resultant modulation of the membrane structure and fluidity (Gelderblom *et al.*, 1996a, Gelderblom *et al.*, 1997, Riedel *et al.*, 2015). Therefore, the effect of FB_1 on lipid metabolism is associated with the progression of various cancers as observed in the studies mentioned above.

2.3.3. FB_1 exposure and effect on maize

Fumonisin B_1 contamination commences with infection of the maize plant, which may occur throughout many of the developmental stages of plant growth (Bacon *et al.*, 2008). However, infection through the silks is the most important pathway and main source of entry (Figure 2) (Cao *et al.*, 2013). After insect injury, fungal propagules are distributed during the feeding and proliferating process. Wounds on the ear tissue may also become infected with already present microconidia or mycelia; resulting in poor stand establishment, stalk rot and kernel infection (Roucou *et al.*, 2021). Asymptomatic infections are quite common among maize plants, which makes it difficult for agriculturists to identify diseased plants preceding the escalation of infection (Cao *et al.*, 2013, Munkvold and Desjardins, 1997). In humans and animals, the effect of FB_1 on various target organ properties are observed, as it varies within different species.

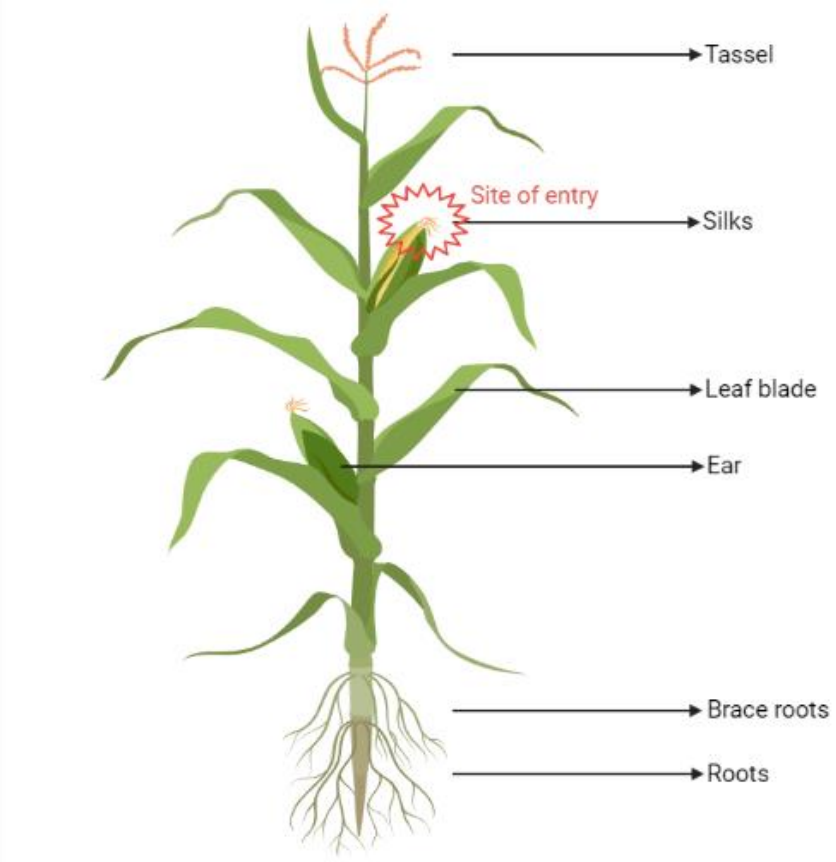

Figure 2: A visual representation of the entry site found in fumonisin contamination on maize, created in BioRender.com

2.3.4. FB_1 exposure and effect on humans and animals

In humans, FB_1 exposure are primarily determined by the agricultural, dietary habits, socio-economical factors, cultural practices and geographical location. For instance, the risk of FB_1 exposure are increased in areas where maize and maize-based products are consumed as a staple diet (Chen *et al.*, 2021). FB_1 is commonly found in areas with increasing occurrences of oesophageal cancer, such as Eastern Cape maize-subsistence farming areas, South Africa (Marasas *et al.*, 1981, Rheeder *et al.*, 1992), Golestan, Iran (Alizadeh *et al.*, 2012), Santa Catarina, Brazil (Van Der Westhuizen *et al.*, 2003); Pordenone, Italy (Franceschi *et al.*, 1990), South Carolina, USA (Sydenham *et al.*, 1991), and parts of China (Braun and Wink, 2018, Chu and Li, 1994, Wang *et al.*, 2000). Additionally, consumption of FB_1 contaminated maize in the early weeks of pregnancy have been identified as a potential risk factor for neural tube defects (NTDs) (Gelineau-van Waes *et al.*, 2009). Increasing NTD cases have also been observed in areas where maize consumption is high (Marasas *et al.*, 2004). Furthermore, it was found that chronic ingestion of contaminated maize (exceeding the provisional maximum tolerable daily intake of 2 µg/ kg body weight per day) has been associated with growth retardation in infants within low-income countries (Kimanya *et al.*, 2010, Shirima *et al.*, 2015).

In animals, FB_1 was found to have species-specific target organ toxicity properties. In swine, ingesting FB_1 caused pulmonary edema and hydrothorax, as well as inducing equine leukoencephalomalacia (ELEM) (Colvin *et al.*, 1993, Harrison *et al.*, 1990, Marasas *et al.*, 1976, Wilson *et al.*, 1990). It was also found to cause liver damage in multiple species, such as swine (Haschek *et al.*, 2001), horses (Ross *et al.*, 1993), cattle (Osweiler *et al.*, 1993), rabbits (Gumprecht *et al.*, 1995), primates (Jaskiewicz *et al.*, 1987), kidneys in rats (Voss *et al.*, 1989), rabbits (Gumprecht *et al.*, 1995) and sheep (Edrington *et al.*, 1995), and being hepatocarcinogenic in rodents (Gelderblom *et al.*, 1988, Howard *et al.*, 2001). Additionally, dietary fumonisin intake have also been associated with reduced weight gain and feed consumption in animals such as swine (Dilkin *et al.*, 2003), rats (Gelderblom *et al.*, 1994), and poultry (Broomhead *et al.*, 2002, Chen *et al.*, 2018, Sharma *et al.*, 2008).

2.3.5. Hydrolyzed fumonisin

Various methods have been established to lessen human and animal fumonisin exposure over the years. One of these strategies is nixtamalization, an alkaline treatment of FB_1 contaminated maize. The tricarballylic acid side chains of FB_1 are cleaved during hydrolysis, and forms

hydrolyzed fumonisin B_1 (HFB_1), also known as aminopentol (AP_1) (Grenier *et al.*, 2012). Similarly, it can be converted to HFB_1 via microbial degradation with the use of FumD, a type-B carboxylesterase presented in Figure 3 (Masching *et al.*, 2016). This observation was only detected in the B-group of fumonisins (Escrivá *et al.*, 2015). The mode of toxicity is similar to FB_1, where HFB_1 also inhibits ceramide synthase, however the potency and cytotoxicity were found to be approximately 10-fold less *in vitro*. A study performed by Schmelz *et al.* (1998), used a human colonic cell line to compare FB_1 and HFB_1 toxicity and found that HFB_1 was less potent, where 50 μM caused the same reduction in cell number compared to 10 μM FB_1. A similar trend was seen *in vivo* where FB_1 fed mice produced hepatocellular apoptosis, hypertrophy, Kupffer cell hyperplasia and macrophage pigmentation. The results of HFB_1 fed mice promoted no alteration in any serum analytes, organ weights or hepatic structure (Howard *et al.*, 2002). Additionally, a study performed by Gelderblom *et al.* (1993) used a rat liver cancer initiation model with dietary fumonisins, where HFB_1 failed to initiate cancer, which suggests a lower toxicity (Humpf and Voss, 2004). Conversely, it was found to be more cytotoxic than the parent molecules in primary rat hepatocyte cultures. Humpf *et al.* (1998) found that HFB_1 not only inhibits ceramide synthase, but is also used as a substrate that is acylated (with fatty acyl- CoA's) to its corresponding N-acyl HFB_X derivative and was found to be more cytotoxic to HT29 cells in culture, compared to HFB_1 (Humpf and Voss, 2004). This suggests that HFB_1 can become more cytotoxic under certain conditions. However, those conditions are poorly understood.

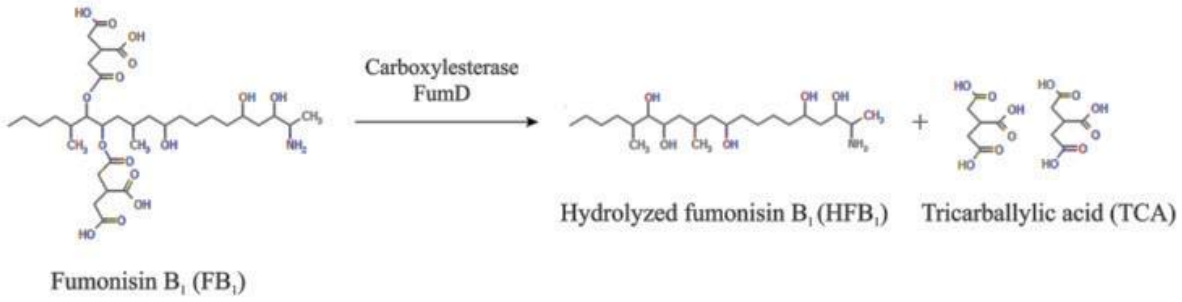

Figure 3: A reaction representing FB_1 converting to HFB_1 using microbial degradation, taken from (Masching et al., 2016)

2.3.6. Fumonisin exposure and stunting

The association between weight reduction and dietary fumonisin exposure in world regions where young children consume maize-based food products have been increasing (Chen *et al.*, 2018). The mechanism by which mycotoxins such as FB_1 promote growth retardation are not well

understood. Initial interaction of mycotoxins after ingestion occurs within the gastrointestinal tract (GIT), specifically the intestinal mucosa. It is suggested that mycotoxins induce enteropathy and dysbiosis by decreasing the nutrient absorption capacity within damaged intestinal mucosa, and that fumonisins act on and break down complex sphingolipid pathways which disrupt barrier integrity through modification of cytokine production and intestinal barrier function with decreased nutrient uptake (Bouhet *et al.*, 2004, Chen *et al.*, 2022, Smith *et al.*, 2015). Environmental enteropathy (EE) in particular is a subclinical condition presented as villous atrophy, crypt hyperplasia and nutrient malabsorption, affecting and promoting growth retardation of children (< 5 years old) in poverty-stricken areas via ingestion and exposure of contaminated food (Ali *et al.*, 2016). Growth retardation occurs due to malabsorption of nutrients in the small intestine and chronic systemic immune activation (Smith *et al.*, 2012). Following ingestion, the GIT is compromised as the intestinal barrier tries to protect the host against harmful mycotoxins (Akbari *et al.*, 2017). Similar trends of reduced weight gain and feed consumption have been identified in animals and plants exposed to fumonisins as mentioned in section 2.2.4. One study reported that infants from Tanzania who ingested maize containing fumonisin exceeding the provisional maximum tolerable daily intake (2 µg/ kg body weight per day) were significantly shorter and lighter than infants who ingested maize with no fumonisin or fumonisin amounts within the tolerable range (Kimanya *et al.*, 2010). Mycotoxins such as fumonisin, when ingested in moderate or high amounts may also result in impaired immunity (Corrier, 1991). The intestinal layer is the first barrier preventing foreign antigens, such as food proteins, natural toxins, commensal gut flora and pathogens from entering. Therefore, barrier integrity is a crucial element in preventing mycotoxin exposure (Bouhet and Oswald, 2005). However, more research must be completed in order to fully understand the mechanisms and interactions between fumonisin, the gut, growth retardation and immunity.

2.4 The small intestinal tract

2.4.1 The structure and function of the gastrointestinal tract

The GIT is a muscular tube within humans and animals, connected by several organs such as the oral cavity, oesophagus, stomach, liver, small intestinal tract, large intestinal tract, and anus. The primary functions of the gut include food ingestion, storage, and digestion, as well as providing barrier protection. It also aids in transporting and absorbing electrolytes, water and nutrients of cells; promoting sufficient immune response and eliminating waste products (Helander and Fändriks, 2014, Liew and Mohd-Redzwan, 2018). It is a continuous tube that consists of four primary layers (Figure 4). The outermost layer is the serosa which consists of

connective tissue, blood vessels, nerves and fat. Beneath this layer lies the muscularis propria, comprised of smooth muscle and aids in peristalsis. The submucosal layer is abundant in arteries, veins, inflammatory cells, lymphatics and autonomic nerves. The innermost layer is the mucosa which is made up of three additional layers namely the epithelium, lamina propria and muscularis mucosae, which are densely folded to increase surface area and aids in nutrient absorption (Liew and Mohd-Redzwan, 2018). These layers are arranged with many organisational and interactive properties which help preserve gut homeostasis (Farré *et al.*, 2020).

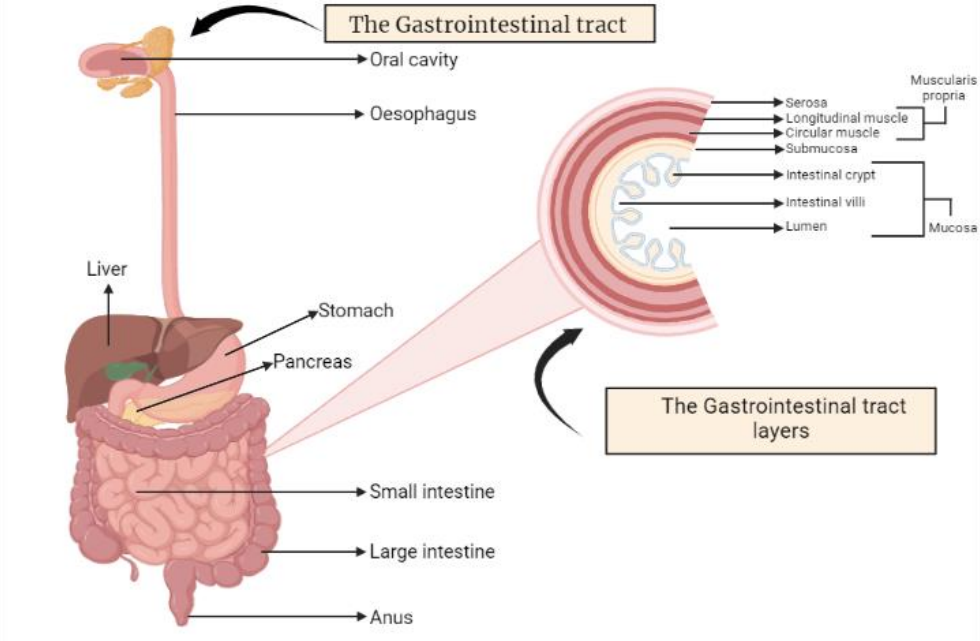

Figure 4: A labelled diagram illustrating the human gastrointestinal tract organs and layers, created in BioRender.com

2.4.2 The intestinal epithelium and barrier integrity

The intestinal epithelium is an organized monolayer of tightly linked columnar epithelial cells with the surface area of approximately 300 m^2 (Günther *et al.*, 2013). It maintains the absorptive, digestive, and secretive capacity of the intestinal tract. The presence of villi and microvilli aids in absorptive capacity and the tightly linked structure is critical for barrier and immune function (Subramanian *et al.*, 2020). The plasma membrane on the epithelium consists of many receptors (including toll-like receptors) which responds to inflammatory and oxidative stress stimuli through bacterial toxins and other proinflammatory cytokines (Lee *et al.*, 2018). These tissues are

constantly being targeted by potential pathogens such as bacteria, fungi, viruses, and parasites through ingestion and needs essential protective agents to prevent invasion and maintain integrity (Hooper, 2015). Intestinal epithelial cells (IEC) separate the lumen from the lamina propria and are formed by stem cells within crypts as shown in Figure 5 (Günther *et al.*, 2013). The mechanism by which cells differentiate, migrate and shed are poorly understood although it is highly regulated (Günther *et al.*, 2013). Several cell types such as absorptive enterocytes, hormone secreting enteroendocrine cells, antimicrobial peptide producing paneth cells, and mucin producing goblet cells are present within the intestinal epithelium. Enterocytes are the primary cells present within the intestinal epithelial layer (> 80 % of the epithelium) and have a high rate of apoptosis to uphold its rapid regenerative capacity (Chassaing *et al.*, 2014). The simple columnar epithelial cells are important in absorption and transport of nutrients from the lumen into the blood stream (Gao *et al.*, 2020, Kong *et al.*, 2018). Like many other epithelial cells, enterocytes assist in the physical and chemical barriers by consisting of a mucus layer and possessing tight junctions. It also has an active role in defending epithelial surfaces by working with specific immune cells to detect and identify foreign and toxic microbes within the lumen present after digestion (Hulst *et al.*, 2019). Enterocytes have the ability to kill bacteria through antimicrobial secretion, and also assists autophagy which produces cytokines and regulate immune responses from sub-epithelial tissues (Hooper, 2015). Majority of these differentiated cell types mature as they travel up the crypt-villus axis, however, paneth cells remain at the base of the crypts and assist with innate mucosal immunity (Campbell *et al.*, 2019, Farin *et al.*, 2012, Gao *et al.*, 2020, Natividad and Verdu, 2013). In this regard, it is evident that the main function of the intestinal epithelium is to provide a first-line defence against foreign substances. This overall structure together with a protective mucus membrane represents the largest barrier protection against external components.

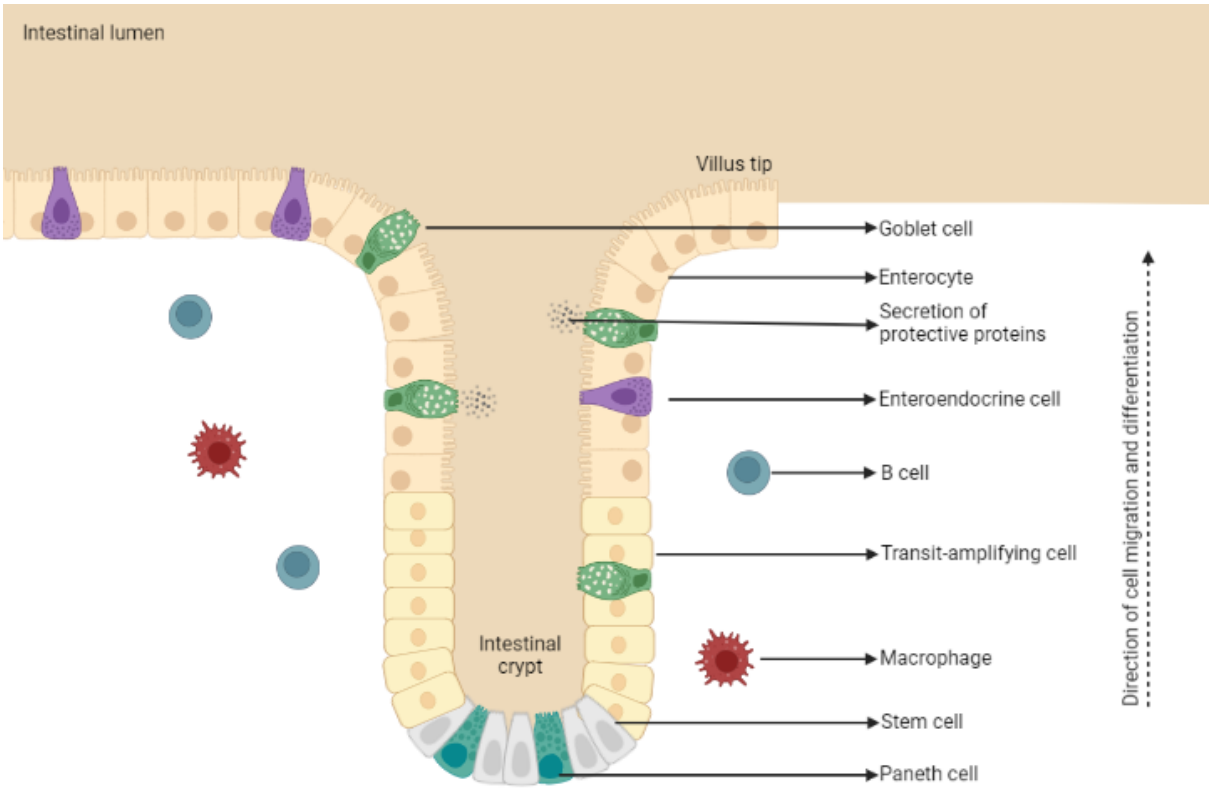

Figure 5: A labelled representation of the intestinal crypt villus which is made up of stem cells that migrate and differentiate into various intestinal epithelial cells such as enterocytes, goblet cells, enteroendocrine cells and Paneth cells. The crypt is also surrounded by immune cells such as B- cells and macrophages (Image created in BioRender.com).

Maintaining barrier integrity is therefore an important factor for healthy gut function and is achieved when the intestinal epithelium, immune system and microbiome works coherently to uphold sufficient homeostasis within the gut (Günther *et al.*, 2013) . The intestinal barrier not only has a physical barrier, but also contains chemical, immunological and microbial barriers for protection against foreign substances, presented in Figure 6. The physical barrier is a tightly connected single layer of IEC, regulated by the apical junction complex (desmosomes, adherens junctions and tight junctions) on the plasma membrane (Ivanov *et al.*, 2010). The apical junction complex allows permeable and/ or semi-permeable interactions of substances via transcellular and paracellular routes (Williams *et al.*, 2015). The chemical barrier is balanced by a variety of elements such as acidity (pH), detergents (bile salts), proteolytic enzymes (trypsin), cell wall degrading enzymes (lysozymes) and antibacterial proteins (defensins) (Chassaing *et al.*, 2014). It is formed by the mucus layer of antimicrobial proteins which blocks the luminal bacteria from the intestinal epithelium (Hooper, 2009, Natividad and Verdu, 2013). The immunological barrier is represented by immune cells (macrophages, B cells, T cells, and dendritic cells) and mediators (secretory immunoglobulin A and cytokines) present within the lamina propria (Andrade *et al.*,

2015). The microbiota barrier is colonized by various commensal bacteria which maintains intestinal health as microbial dysbiosis often leads to intestinal inflammation (Gao *et al.*, 2020).

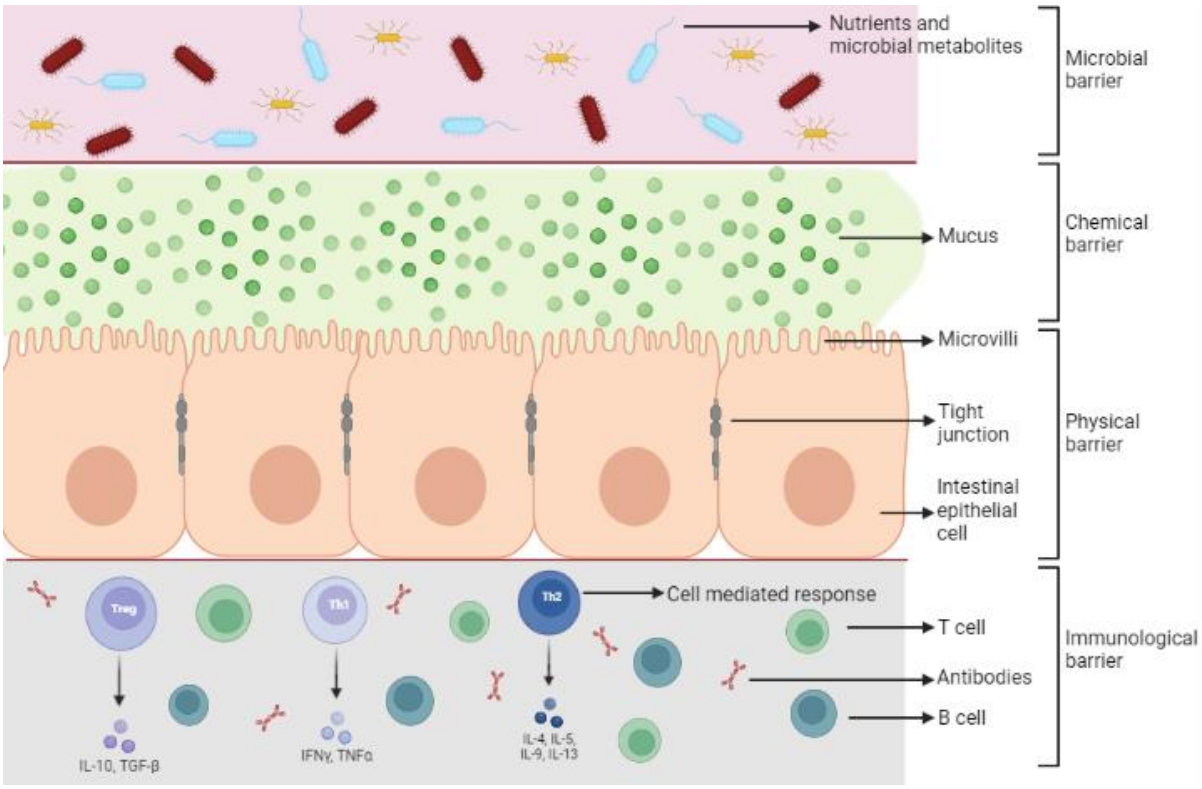

Figure 6: A graphical representation of the four major barriers found within the intestinal epithelium, created in BioRender.com

2.4.3 Gut homeostasis and injury

Optimal barrier integrity leads to efficient homeostasis within the intestinal epithelium (Günther *et al.*, 2013). Any adverse effects or alterations upon these properties contributes to gut injury and impaired health (Subramanian *et al.*, 2020). Organ damage is commonly associated with cytokine and endotoxin release from the gut and is a well-known indicator for intestinal injury or barrier integrity disruption (Armacki *et al.*, 2018). Intestinal inflammation is often the observable link between pathogen invasion and immune response within the epithelium (Ivanov *et al.*, 2010). It compromises the protective barrier and allows the invasion of pathogens through various entry sites. Pathogens may invade via the luminal side of the epithelium by increasing the epithelial permeability through the secretion and release of epithelial barrier disrupting agents (pore-forming

toxins, cytoskeleton modifying proteins, and bacterial lipopolysaccharides). Additionally, it may invade from the tissue side where mucosal immune cells also increases the epithelial permeability through proinflammatory cytokine secretion. The secretion of distinct cytokines along with the release of proteases and reactive oxygen species (ROS) occurs with inflammation and are identified as common markers (Ivanov *et al.*, 2010).

2.4.4 Cytokines as markers for gut injury and inflammation

Cytokines such as interleukins, tumour necrosis factors, interferons, transforming growth factors and chemokines are soluble regulating agents of inflammation and immunity (Stadnyk, 2002). It affects gut homeostasis and are often important markers and communicators of acute and chronic inflammatory related injuries (Andrews *et al.*, 2018). Cytokines that are commonly released alongside inflammation are interleukin 6 (IL-6), interleukin-1β (IL-1β), tumour necrosis factor α (TNF-α), interferon gamma (IFNγ), transforming growth factor β (TGFβ), and a chemokine interleukin 8 (IL-8) which are all predominantly produced by macrophages and monocytes at the inflammatory site (Gabay, 2006). For example, when the intestine is inflamed, cytokines such as IL- 6, TNF-α, IL-18, IL-1β and interleukin-17 (IL-17) are overexpressed (Neurath, 2014). Cytokines have the ability to directly alter intestinal epithelial permeability through modification of tight junction permeability (Andrews *et al.*, 2018). Cellular chemokine release also promotes destructive invasion of immune cells into essential organs and tissues (Turner *et al.*, 2014). Cytokines transport essential immune cells to the area of inflammation where the rate of cellular death and/ or survival depends on the inflammatory destruction occurred. Among their many functions and effects, cytokines and chemokines are also crucial elements in upholding barrier integrity (Andrews *et al.*, 2018). The following are important cytokines affecting barrier integrity:

2.4.4.1. Tumour necrosis factor alpha

Tumour necrosis factor (TNF-α) is a pleiotropic cytokine that plays an important role in the innate immune system by inducing cytokine production, activating adhesion molecules and growth stimulation (Rothe *et al.*, 1992, Tartaglia and Goeddel, 1992). It is largely secreted from activated macrophages and stimulates the proliferation of normal cells, as well as causing inflammatory, antiviral and immunoregulatory effects on tumour cells during cytolytic or cytostatic exertion (Tracey *et al.*, 2008). Furthermore, TNF-α regulates many cellular processes such as lipid metabolism, coagulation, insulin resistance and endothelial function which makes it a potent mediator of intestinal inflammation, immune and apoptotic responses (Turner *et al.*, 2014). These

factors are closely linked to epithelial injury responses, therefore making it a crucial regulator of the intestinal barrier (Leppkes *et al.*, 2014).

2.4.4.2. Interleukin 6

Interleukin 6 (IL-6) is also a pleiotropic cytokine, commonly expressed by mononuclear phagocytes, immune cells and other connective tissue cells (Jucker *et al.*, 1991). It promotes final maturation of B-cells into antibody-producing plasma cells, additionally maintaining T-cell activation and differentiation. IL-6 aids in the secretion of acute phase proteins by the liver together with interleukin-1 (IL-1) and is largely secreted from IECs and laminar propria mononuclear cells from patients with active inflammatory bowel disease (Turner *et al.*, 2014, Wang *et al.*, 2003).

2.4.4.3. Interleukin 8

Interleukin 8 (IL-8/ CXCL8) is an important inflammatory mediator (chemokine) that acts as an angiogenic factor in endothelial cells. It recruits neutrophils while also migrating and activating inflammatory cells at inflammation sites (Turner *et al.*, 2014). The presence of live bacteria, lipopolysaccharides (LPS) and early proinflammatory cytokines induces the secretion of IL-8, which is mainly produced by monocytes and macrophages. IL-8 is often excreted into the extracellular space and is usually produced shortly after inflammatory exposure. Moreso, IL-8 resists high temperatures and acidic environments, as well having an increased longevity rate. Thus, making it ideal to be produced in acute inflammatory environments, such as the intestinal epithelium (Remick, 2005).

The health of many cells is controlled by various cytokines and directly affects cellular processes such as cell proliferation and cell death within the intestinal epithelium. These processes are constantly regulated by multiple cytokines to induce or restrict certain IECs which maintain barrier homeostasis (Andrews *et al.*, 2018). Furthermore, it is also important to note that cytokines maintaining the intestinal barrier integrity may also be key regulators of cell death (Sharma and Anker, 2002). For example, cytokine induction within the gut may promote an increase in intestinal epithelial cell death while also disrupting the intestinal barrier integrity (Andrews *et al.*, 2018, Subramanian *et al.*, 2020).

2.5 Apoptosis on cell barrier function

Apoptosis or programmed cell death is a genetically controlled process when cells undergo controlled cell death, which prevents a spillage of cell remnants into the surrounding cellular environment (Kerr *et al.*, 1972). Upon cell damage detection, a number of processes are controlled by a family of protease enzymes called caspases. Inactive caspase precursors, called procaspases are activated by initiator caspases (caspase 8 and 9) which subsequently activates executioner caspases (caspase 3, 6 and 7). This process results in deoxyribonucleic acid (DNA) fragmentation, destruction of nuclear proteins and cytoskeleton, crosslinking of proteins, and the expression of ligands for phagocytic cells. It also forms apoptotic bodies, which are phagocytosed by the surrounding cells (D'Arcy, 2019, Subramanian *et al.*, 2020). Initiation may occur through the intrinsic (apoptosis initiation through cellular damage identification via intracellular sensors) or extrinsic (apoptosis initiation through cellular damage identification via the immune system) pathways (D'Arcy, 2019). Apoptosis is important in sustaining sufficient homeostasis within cells, as insufficient or increased cell death leads to pathological cell shedding (Williams *et al.*, 2013). For instance, enterocytes have a high rate of apoptosis which supports faster replacement of compromised cells, subsequently preventing compromised barrier integrity (Chassaing *et al.*, 2014).

Additionally, there are multiple regulated points where proteins can be evaluated within the entire apoptosis process. Many assays are used to identify various endpoints associated with various apoptotic phases. For example, certain assays may only be able to identify certain biomarkers at the initiation of apoptosis whereby another may only be able to detect biomarkers affected towards the end of the apoptosis process (Elmore, 2007). Researchers would have to include multiple assays in order to fully understand which cytokines and/ or proteins are affected at various points of the apoptotic process. Recently, high-throughput screening methods have been incorporated into research experiments to identify a spectrum of biomarkers that may be affected by a particular stimulant. Therefore, many researchers are advancing towards proteomics research (Tyers and Mann, 2003).

2.6 Proteomics

2.6.1 Overview

There have been many advances in molecular methodology related to understanding disease pathogenesis over the years, yet significant gaps in research still remain. Recently, the focus on experimentation have shifted towards analytical methodology, which uses highly specialized instrumentation in order to determine the physical and /or chemical composition of a specific sample.

Proteomics in particular, is the attempt to identify and quantify proteomes, three-dimensional (3-D) protein structures, and protein interactions within an organism (Cho, 2007, Kenyon *et al.*, 2002). Proteins are necessary for many biological processes and provide sufficient structural integrity in cells. They also play a key role in metabolism, bio-signalling, gene regulation, protein synthesis, solute transport, and immune function. Proteins can be described through many features, such as expression, localization, interaction, domain structure, modification, and specific activity (Graves and Haystead, 2002, Han *et al.*, 2008). The proteome, coined by Wasinger *et al.* (1995), Wilkins *et al.* (1996a), is the entirety of proteins expressed from its corresponding genome within a specific cell or tissue. The presence of the proteome indicates the product obtained directly from the genome; however, the proteome can have a greater number of proteins compared to the number of genes present through alternate gene splicing (Wilkins *et al.*, 1996b). In this regard, the proteome provides a greater understanding of the immunomodulatory and regulatory mechanisms as well as the functions present in the desired cell or tissue. Additionally, contributing to a broader range of data pertaining to biological activities within these tissues (Kim *et al.*, 2016). For example, in epithelial cells the information obtained from culture and immunoassays, although sufficient in understanding the basic principles of interactions, are still quite limited when compared to obtaining the proteome and discovering protein interactions. The sensitivity of data obtained from proteomics far exceeds that of viability or immunoassays. The presence of 3-D structures promotes a visual representation of where proteins are located. Subsequently, providing specific protein networks within various cells and tissues, allowing for identification of various cellular functions (Graves and Haystead, 2002). With regard to fumonisin contamination, the use of proteomics will provide a better understanding of the underlying mechanisms at both genetic and translational levels and will be able to show exactly how fumonisin interacts with various tissues.

These observations may only be obtained through highly sensitive and specialized techniques such as mass spectrometry (MS), two-dimensional (2-D) gel electrophoresis, two-hybrid analysis, protein microarrays, and cell imaging. Although the above techniques are capable of identifying and quantifying proteomes, MS exceeds in terms of throughput efficiency alongside highly sensitive complexities during proteome investigations. Proteomic MS allows the application of multiple methodologies instead of a single technique to identify and quantify proteins (Han *et al.*, 2008). There are two distinct methods in which protein identification occurs through MS. The first method being whole-protein level analysis (also known as 'top-down' proteomics). During this process, whole protein ions are placed into the gas phase by electrospray ionization and subsequently fragmented via collision-induced dissociation, electron-capture-dissociation, or electron-transfer dissociation within the MS. The product is the mass of both protein and fragment ions which are compared and identified from databases through the use of search engines (Catherman *et al.*, 2014, Timp and Timp, 2020). The second and more common method of protein identification through MS are enzymatically or chemically produced peptide analysis (also known as 'bottom-up' proteomics). During this process, proteins are digested by enzymes prior to MS analysis. The resulting peptides are then ionized, and separated according to their mass/charge ratio (m/z). The peptides are further ionized by electrospray ionization or matrix-assisted laser desorption ionization (MALDI) and conveyed into the MS. Thereafter, peptide masses are compared with known proteins in a database using search engines (Chait, 2006, Timp and Timp, 2020).

Database comparisons are performed using bioinformatics applications. Bioinformatics have been used more commonly in many biomedical studies and are gradually increasing. The term bioinformatics is represented through the use of computational techniques related to biological macromolecules. Analysis focuses on data such as DNA or protein sequences, macromolecular structures and the results of functional genomic experiments. These data sources are usually organised through bioinformatics analysis, which then becomes easily available to researchers who intend to access or expand on existing data. The use of bioinformatics promotes an advancement of tools and resources for said data sources, subsequently applying the tools to analyse and interpret large datasets, accordingly (Luscombe *et al.*, 2001). These methods, although complicated, are the key to obtaining the proteome of a particular cell or tissue and promote similar challenges to that of genome identification (Wilkins *et al.*, 1996b).

MS instrumentation allows for sufficient protein identification and information about the type and location of proteins being produced within a particular cell or tissue. The distinct method of

isolating and identifying proteins through this technology makes each individual experiment unique as the methodology and instrumentation are altered according to specific criteria (Han *et al.*, 2008). Additionally, the identification of proteins obtained may provide information about the desired experimentation, such as an unexpected result that differs from the results obtained from less sensitive techniques. It may also reveal the reason why specific biochemical methods may not be working or need altering (Graves and Haystead, 2002). Hence, proteomic data obtained after MS and bioinformatics analysis are gradually becoming a necessity in many fields of study.

In conclusion, the overall effect of mycotoxins such as FB_1 and HFB_1 on intestinal health have been studied exponentially over the years. The recent incorporation of omics technology into current experimental model provides a better understanding of the underlying mechanism of these toxins on intestinal cell lines and overall gut health. Additionally, considering the effect of toxins in cohesion with endotoxins, such as LPS, within these cell lines simulates the effect of humans becoming infected by these toxins while already being compromised with intestinal inflammation. Therefore, in this study, the effect of FB_1 and HFB_1 within a LPS inflammatory model, was observed on gut integrity and immunity of the intestinal porcine enterocyte (IPEC-J2) cell line. The protein cell signalling pathways were obtained through omics technology and aims to provide a better understanding of intestinal-mycotoxin cellular interactions.

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CHAPTER 3: Fumonisin B_1, Hydrolyzed fumonisin B_1, and Lipopolysaccharide Model Development for the IPEC-J2 Cell Line

ABSTRACT

A substantial amount of staple feed and food in especially low- and middle-income countries are being exposed various mycotoxins, such as fumonisins. One of the most toxic and abundant fumonisins found in nature, fumonisin B_1 (FB_1) is also one of the most common maize-contaminating mycotoxins. FB_1 can be converted to hydrolyzed fumonisin B_1 (HFB_1), by a microbial carboxylesterase. In the present study, the modulating effect of FB_1, HFB_1 and lipopolysaccharides (LPS) on the growth and immune indices of the intestinal porcine enterocyte (IPEC-J2) cell line was investigated. The cell survival indices for individual and co-exposure treatments: cell viability, apoptosis, and cell proliferation were measured; while inflammatory responses were monitored by immune-detection of interleukin 8 (IL-8). It was found that HFB_1 decreased cell viability and cell proliferation at concentrations above 250 µM in a dose-dependent manner after 24 hours of exposure, stimulating apoptosis. However, no IL-8 was detected at these concentrations. Lower concentrations of HFB_1 and all FB_1 concentrations showed no effect on cell viability, producing a slight elevation of IL-8 and caspase-3 activity. However, a decrease in cell proliferation of FB_1 concentrations was observed. Individually and concurrent with LPS exposure, FB_1 exposed cells (7.81 µM and 15.63 µM concentrations) presented no significant effects on cell viability, cell proliferation and IL-8 concentration after 24 hours. Individual 15.63 µM HFB_1 exposure presented an increase in apoptosis, and a decrease in cell proliferation and IL-8 concentration, similar to the individual concentrations observed. However, combined with LPS, HFB_1 exposed cells promoted a slight increase in cell viability and IL-8 concentration, and a decrease in apoptosis, promoting an increased toxicity with the addition of LPS. Individually, it is noted that HFB_1 promotes a greater toxicity on intestinal epithelial cells *in vitro* compared to FB_1. Although HFB_1 is known to be less toxic compared to FB_1 *in vivo*, which suggests that HFB_1 is metabolized differently *in vitro* compared to *in vivo*.

3.1 Introduction

An emerging concern relating to food safety and security within low- and middle-income countries are increasing (Grace, 2015). In certain areas staple feed and food are known to be contaminated by various mycotoxins, due to poor agricultural practices. This is especially true in subsistence farming areas reliant on a daily staple of maize and maize-based food products (Bryden, 2012, Maresca and Fantini, 2010).

One of the most toxic and abundant fumonisins found in nature, fumonisin B_1 (FB_1) is also one of the most common maize-contaminating mycotoxins (Bouhet *et al.*, 2004). FB_1 toxicity targets various organs of farm and laboratory animals, while in humans is it has been linked to a higher risk of neural tube defects, liver tumours, and oesophageal cancer (Degen, 2015, Warth *et al.*, 2016). Pigs amongst other animals are the most susceptible to FB_1, with increased exposure to FB_1-contaminated food and feed leading to various heart, lung, and liver diseases (Harrison *et al.*, 1990, Haschek *et al.*, 2001). FB_1 may also undergo a detoxification pathway that results in a less potent ceramide synthase inhibitor called hydrolyzed fumonisin B_1 (HFB_1) (Humpf *et al.*, 1998, Schelstraete *et al.*, 2020). Additionally, prolonged exposure to both FB_1 and HFB_1 have been reported to cause adverse effects on the intestinal functions of animals ingesting these mycotoxins at increased concentrations (Alassane-Kpembi and Oswald, 2015, Ghareeb *et al.*, 2015, Grenier and Applegate, 2013). HFB_1 toxicity in animals and humans is somewhat controversial due to it resulting in a lower toxicity in many *in vivo* models, while causing an equal or increased toxicity to FB_1 (the parent molecule) in others (*in vivo* and *in vitro* models). However, the molecular mechanisms of the toxicity exerted have not yet been fully understood (Caloni *et al.*, 2005, Dellafiora *et al.*, 2018, Hartl and Humpf, 2000, Wang *et al.*, 2016b).

Recently, the focus has shifted to include more *in vitro* studies to understand exactly how FB_1 and HFB_1 interact with various cell lines, especially within the gastrointestinal tract. Cell lines such as intestinal porcine enterocytes (IPEC-J2) are highly suitable for these types of analysis, as the intestine is the first target for mycotoxins following ingestion of contaminated feed (Schierack *et al.*, 2006). Intestinal cells are reported to be highly sensitive to FB_1 and HFB_1 as prolonged exposure may lead to a decrease in barrier integrity, promoting an increase in bacterial translocation across the intestine, which increases susceptibility to various enteric infections, sepsis and inflammation (Gao *et al.*, 2020, Pierron *et al.*, 2016). Additionally, a disruption in the intestinal barrier specifically may also disturb junctional movement between the intestinal barrier itself and the circulation, causing an influx of cytokines from macrophages, which subsequently

increases inflammation within the intestine (Ghosh *et al.*, 2020). Chronic increase of inflammatory mediators within the gut may cause a greater disruptive effect on the intestinal barrier, and promote the initiation of pathological processes which may also lead to various chronic diseases or disorders (Fasano and Shea-Donohue, 2005, Groh *et al.*, 2017, Hakansson and Molin, 2011, Kurashima *et al.*, 2013). In this regard, the development of tumours is possible and may be impacted by the gut microbiota as increasing tumours are generally associated with pro-inflammatory environments (Al Bander *et al.*, 2020, Singh *et al.*, 2019, Wang and Huycke, 2007). This poses an additional risk to animals and humans chronically exposed to FB_1 as it is a group 2B carcinogen (Guo *et al.*, 2020, IARC, 2002). The inflammatory-related diseases and disorders as well as the potential for tumour progression are increasing. Therefore, the individual as well as the co-occurring effect with other known contaminants of FB_1 have to be fully analysed to fully understand the interactions occurring within the gut.

In addition to interacting with other mycotoxins during pre- and post-harvest practices, FB_1 may also have an interactive effect with various environmental contaminants, such as soil, water, air, plants, animals, food and feed as well (Guo *et al.*, 2020). This also includes many natural toxins, such as fungal toxins, bacterial toxins, phytotoxins and algal toxins (Mol *et al.*, 2008). Recently, it has been observed that bacterial toxins are one of the most common contaminants to interact with mycotoxins. Lipopolysaccharides (LPS), a bacterial toxin (also known as endotoxin), is one of many important structures called inflammation-inducing pathogen association molecular patterns (PAMPs), which activate and induce the release of pro-inflammatory mediators (cytokines) through pattern recognition receptors (Frank *et al.*, 2016, Gong *et al.*, 2020, Janeway, 1989, Kawai and Akira, 2006). Mycotoxin interactions with LPS have shown to induce immune and inflammatory responses (Guo *et al.*, 2020). However, the molecular mechanisms from FB_1, HFB_1 and LPS interactions have not yet been fully understood.

The current chapter focuses on the individual and co-occurring preparation, characterization, and effect of FB_1, HFB_1 and LPS on the porcine intestinal cell line, IPEC-J2.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Cell culture materials, Dulbecco's Modified Eagles medium-Ham's F-12 (DMEM HAMS F-12), fetal bovine serum (FBS), epidermal growth factor (EGF), and insulin-transferrin-selenite (ITS) were obtained from Gibco-Life Technologies (Paisley, UK). L-glutamine, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer, trypsin, Hank's buffered salt solution (HBSS), Penicillin-Streptomycin-Amphotericin B (Pen/Strep/Amph), and Dulbecco's Phosphate Buffered Saline (DPBS) were obtained from Lonza (Basel, Switzerland). Tissue culture flasks, clear 96-well flat bottom and high binding enzyme linked immunosorbent assay (ELISA) mictrotiter plates (MPs) were obtained from Lasec (Cape Town, South Africa). White 96-well MPs were purchased from Corning Incorporated (Maine, USA), and black MPs from Whitehead Scientific (Cape Town, South Africa).

The porcine IL-8, IL-6 and TNFα ELISA kits, normal goat serum, stop solution, substrate solution, and wash buffer were all purchased from R&D systems (Minneapolis, USA). CellTitre-Glo luminescent cell viability and caspase 3/ 7 assay kits were obtained from Promega (Madison, USA). The cell proliferation ELISA, 5'bromo-2'-deoxyuridine (BrdU) and Triton X-100 were purchased from Roche (Mannheim, Germany), and bovine serum albumin (BSA) from Biowest (Nualle, France).

Fumonisin B_1 and hyrolyzed fumonisin B_1 were supplied by the Applied Microbial and Health Biotechnology Institute (AMHBI) (Cape Town, SA). Dimethyl sulfoxide (DMSO) and staurosporine were obtained from Sigma-Aldrich (Missouri, USA), and lipopolysaccharide (LPS) from MERCK (Darmstadt, Germany).

3.2.2 Cell line and cell cultures

3.2.2.1 Intestinal porcine enterocytes

Non-transformed, secondary intestinal porcine enterocytes (IPEC-J2) were gifted from Dr Elisabeth Mayer, University of Innsbruck (Biomin, Tulln, Austria). These cells were originally isolated from the jejunal epithelium of neonatal un-suckled piglets. Cells were inoculated in DMEM HAMS F-12 revival media (supplementation provided in appendix 1) and maintained in supplemented maintenance media (supplementation provided in appendix 1). Thereafter, the cells were passaged twice a week at a split ratio of 1:3 and incubated at 37 ℃ in humidified air containing 5 % carbon dioxide (CO_2)/ 95 % air.

3.2.2.2 Cell seeding preparation

Upon reaching 70-80 % confluency, cells were washed with HBSS, trypsinated and seeded (200 µL) in supplemented maintenance media at the density of 3×10^4 cells per well in solid white, clear flat-bottom, and black 96-well tissue culture MPs. These were used to determine the adenosine triphosphate (ATP), caspase-3/7, and BrdU content respectively. Cells were incubated for a minimum of 24 hours at 37 ℃ in 5 % CO_2/ 95 % air, before media was discarded, and treated (100 µL) with the desired concentrations of FB_1, HFB_1 and LPS, made up in 0.5 % DMEM HAMS F-12 media with 1 % DMSO (additional supplementation provided in Appendix 1). The control well contained only 0.5 % FBS DMEM/ HAMS F-12 media with 1 % DMSO, and 200 nM staurosporine was used as the positive control. Cells were treated with FB_1, HFB_1 and LPS for 6 and 24 hours before commencing with further analysis. Each experiment was repeated at least twice, using five replicates of each concentration of treatment. The above individual concentrations were then analyzed and optimized for the co-exposure model. The chosen concentrations of FB_1, HFB_1, and LPS used in the co-exposure model are provided in Table 2.

Concerning the co-exposure model, cells were prepared and seeded at the density of 3×10^4 cells per well in solid white, clear flat-bottom, and black 96-well tissue culture MPs as mentioned above. These were used to determine the ATP, caspase-3/ 7, and BrdU content, respectively. Cells were then treated with the desired combination concentrations of FB_1 and HFB_1 with LPS provided in Table 3, and incubated for 24 hours before commencing further analysis. For each experiment, five replicates of each treatment concentration were prepared in duplicate.

3.2.3 Treatment preparation of FB_1 and HFB_1

Purified and extracted FB_1 (> 95 %) and HFB_1 (> 98 %) were weighed (21.6 mg/ mL and 5 mg/ mL respectively) and dissolved in DMSO forming a stock solution of 1 mg/ mL. The final DMSO concentration did not exceed 1 % for all treatments. For the individual concentrations, a 500 µM concentration of FB_1 and HFB_1 were made up in 0.5 % DMEM HAMS F-12 media with 1 % DMSO (additional supplementation provided in Appendix 1). It was sterilized with 0.22 µM corning syringe filters and diluted into various concentrations provided in Table 2.

3.2.4 Treatment preparation of LPS

A 1 mg/ mL stock solution of LPS was made up of 0.5 % DMEM HAMS F-12 media with 1 % DMSO (additional supplementation provided in Appendix 1), sterilized using 0.22 µM corning syringe filters, and diluted into various concentrations (1.25, 2.5, 5, and 10 µg/ mL).

Table 2: Individual concentrations of FB_1 and HFB_1 used for IPEC-J2 cells exposure

Treatment	Individual mycotoxin concentrations (µM)									
FB_1	0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250	500
HFB_1	0.98	1.95	3.91	7.81	15.63	31.25	62.5	125	250	500

FB_1 and HFB_1 were made up in 0.5 % FBS DMEM HAMS F-12 media with 1 % DMSO. The control was only 0.5 % FBS DMEM HAMS F-12 media with 1 % DMSO and the positive control was 200 nM staurosporine. Five replicates of each concentration of FB_1 and HFB_1 and the controls were made up for each experiment.

Table 3: Co-exposure treatment preparation

Mycotoxin and /or Endotoxin	Concentrations	
FB_1	7.81 µM	15.63 µM
HFB_1	7.81 µM	15.63 µM
LPS	10 µg/ mL	
FB_1 + LPS	7.81 µM FB_1 + 10 µg/ mL LPS	15.63 µM FB_1 + 10 µg/ mL LPS
HFB_1 + LPS	7.81 µM HFB_1 + 10 µg/ mL LPS	15.63 µM HFB_1 + 10 µg/ mL LPS
FB_1 + HFB_1	7.81 µM FB_1 + 7.81 µM HFB_1	15.63 µM FB_1 + 15.63 µM HFB_1

The final concentrations of FB_1 and HFB_1 with LPS were made up in 0.5 % FBS DMEM HAMS F-12 media with 1 % DMSO and used in co-exposure runs. The controls used were 0.5 % FBS DMEM HAMS F-12 media with 1 % DMSO, the positive control, 200 nM staurosporine, and the individual concentrations of FB_1, HFB_1 and LPS. Five replicates of each concentration were made up for each experiment.

3.2.5 Cell viability indices and Inflammation biomarkers

3.2.5.1 Determination of cell viability (ATP production)

To determine the cell viability of eukaryotic cells, colorimetric assays have been used to quantify various substances within cellular processes. The most routinely-used method to estimate the number of viable cells is the measurement of ATP using firefly luciferase, since ATP has widely been accepted as an effective marker of viable cells. It uses the properties of a stable form of luciferase, to enable reaction conditions that generate a stable luminescent signal while simultaneously inhibiting ATPases. The ATP content or the number of viable cells of the IPEC-J2 cell line were determined using the CellTiter-Glo luminescent cell viability kit according to the manufacturer's instructions (Promega Corporation, Madison, USA). In a solid white 96-well MP, cells were exposed to various concentrations of FB_1, HFB_1 and LPS for 6 and 24 hours. For the co-exposure model, cells were only exposed FB_1 and HFB_1 for 24 hours. After the incubation period, plates were equilibrated for 30 minutes at room temperature in the dark, and an equal volume of luciferase reagent (100 µL) was added to each cell-containing well. Plates were rotated on an orbital shaker at 500 revolutions per minute (rpm) for 2 minutes to induce cell lysis, and incubated in the dark for an additional 10 minutes at room temperature to stabilize the luminescent signal. The signal (expressed as relative light units (RLU)) was recorded using the Veritas microplate luminometer (Turner Biosystems, California, USA), and data were expressed as a percentage of ATP content of control cells. The percentage of cell viability was calculated using the following formula:

$$\% \text{ Viable cells} = \frac{\text{RLU (sample)}}{\text{RLU (control)}} \times 100$$

3.2.5.2 Determination of apoptosis

To determine apoptosis, the caspase-3 and -7 activity within cells that contain the tetrapeptide sequence DEVD was determined. The addition of the caspase reagent promotes cell lysis and subsequently caspase cleavage. Thereafter, a luminescent signal is produced by luciferase, such that luminescence is proportional to the amount of caspase activity present (Promega, 2015). As mentioned in section 3.2.2, cells were treated and incubated in clear flat-bottom 96-well MP's. Thereafter, the cell lysates were lysed with 100 µl of filtered 0.5 % Triton X-100 made up in DPBS and stored at -80 °C until analysis. Caspase-3 and -7 activity were measured using the Caspase-Glo assay kit (Promega Corporation, Madison, USA) according to the manufacturer's instructions. Before analyses, one freeze-thaw cycle was completed along with a two-minute rotation on an

orbital shaker at 500 rpm. The lysed cell lysate (20 µL) was transferred into non-sterile, solid white 96-well MP and an equal volume of caspase 3/ 7 Glo reagent was added. It was rotated on an orbital shaker at 500 rpm for 30 seconds and incubated in the dark at room temperature for an hour. The luminescent signal (RLU) was recorded using the Veritas microplate luminometer (Turner Biosystems, California, USA), and data (representing the caspase-3/ 7 activity within cells) expressed as a fold increase relative to the control. The calculation used to determine Caspase-3/ 7-fold increase was as follows:

$$\text{Capase-3/ 7-fold increase} = \frac{\text{RLU (sample)}}{\text{RLU (control)}}$$

3.2.5.3 Determination of cell proliferation

The cell proliferation ELISA, BrdU chemiluminescent assay was used to observe the effect of FB_1, HFB_1 and LPS on cell proliferation, via the incorporation of BrdU (5'bromo-2'-deoxyuridine) within newly synthesized DNA during replication. All instructions were followed according to the assay specifications. In a solid black 96-well MP, cells were treated and incubated as previously mentioned in sections 3.2.2. Thereafter, cells were labelled with BrdU (10 µL) and reincubated for an additional 2 hours at 37 °C . The media was then discarded, and fixed cells and DNA were denatured by adding FixDenat (200 µL). Cells were then incubated at room temperature for 30 minutes before the antibody, anti-5'bromo-2'-deoxyuridine-peroxidase (Anti-BrdU-POD) (100 µL) was added. It was incubated for an additional 90 minutes and washed with a washing solution (3 x 200 µL) for 5 minutes per wash. Subsequently, a substrate solution (100 µL) was added and then the plate was rotated on an orbital shaker at 500 rpm for 3 minutes. Chemiluminescence signal (RLU) was detected using the Veritas Microplate Luminometer (Turner Biosystems, California, USA), and data expressed as a percentage of cell proliferation over controlled cells. The percentage of cell proliferation was calculated using the following formula:

$$\text{\% Cell proliferation} = \frac{\text{RLU (sample)}}{\text{RLU (control)}} \times 100$$

3.2.5.4 Porcine interleukin-8 ELISA

The IL-8 content in the cell supernatant was determined using a porcine IL-8/ CXCL8 ELISA kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. Cells were treated and incubated as previously mentioned in section 3.2.2, in clear flat-bottom 96-well MP's. Thereafter, the cell supernatants (100 µL) were transferred to sterile clear flat bottom 96-well MP's and stored at -80 °C until analysis. Prior to analyses, one freeze-thaw cycle was completed along

with a 2-minute rotation on an orbital shaker at 500 rpm. Various concentrations of IL-8 standard ranging from 125 – 8 000 pg/ mL were used with porcine IL-8 prepared in filtered 1 % BSA (w/ v) in DPBS to generate a standard curve. Absorbance was measured at 450 nM with the Synergy 2 Multimode Microplate Reader (Biotek®, Vermont, USA) and data were analysed using the standard curve generated from Gen5™ Data Analysis Software (Version 2 for Windows). Extracellular porcine IL-8 was expressed as pg/ mL of cell supernatant and the fold increase of the untreated control (samples not irradiated). The following formula was used:

$$\text{Porcine IL-8 Fold increase} = \frac{Porcine\ IL{-}8\ (sample)}{Porcine\ IL{-}8\ (control)}$$

3.2.6 Statistical analyses

For statistical analysis of individual FB_1, HFB_1 and LPS concentrations, an analysis of variance (ANOVA) was used to test for significant group effects using the Tukey-Kramer multiple-test comparison test procedure. Co-exposure concentrations were subjected to tests on normality and equality prior to analysis. Tests of the normality of residuals assumption were performed using Shapiro-Wilk, Anderson-Darling, D'Agostino Skewness, D'Agostino Kurtosis and D'Agostino Omnibus procedures. The tests of the equality of group variances assumption were performed using Brown-Forsythe, Levene, Conover, and Bartlett procedures. Thereafter data were analyzed using an ANOVA to test for significant group effects using the Tukey-Kramer's all pairs simultaneous confidence intervals of mean difference and *p*-value procedure.

3.3 Results

3.3.1 The effect of fumonisin B_1 and hydrolyzed fumonisin B_1 on cell survival indices of IPEC-J2 cells

3.3.1.1 The effect of FB_1 and HFB_1 exposure on cell viability

To determine the effect of FB_1 and HFB_1 on cell viability, cells were exposed to FB_1 and HFB_1 at various concentrations (0.98 – 500 µM) for 6 and 24 hours (preparation and seeding calculations provided in section 3.2.3). Following 6 hours FB_1 exposure, (Figure 7A and C), a significant ($p < 0.01$) increase in cell viability (< 100 %) was observed at 1.95 µM, 15.63 µM and 31.25 µM concentrations compared to the control. All other concentrations showed no significant differences when compared to the control. Cells that were exposed to HFB_1 for 6 hours exhibited a significant increase ($p < 0.05$) in cell viability (> 110 %) at concentrations 0.98 µM, 7.81 µM, 15.63 µM, 31.25 µM and 250 µM. However, following 6 hours 500 µM HFB_1 exposure, a significant decline ($p < 0.01$) in cell viability (< 90 %) occurred compared to the control.

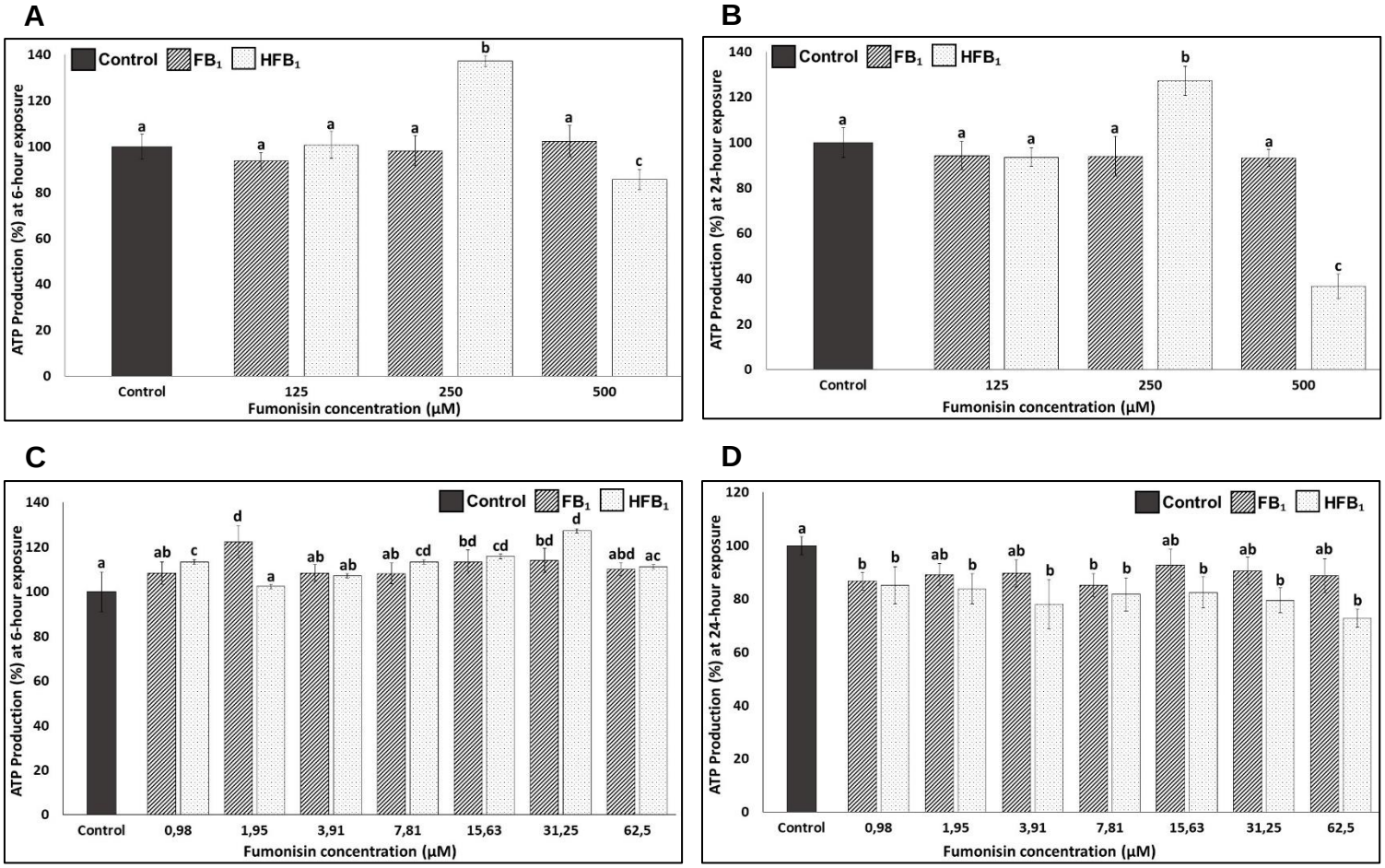
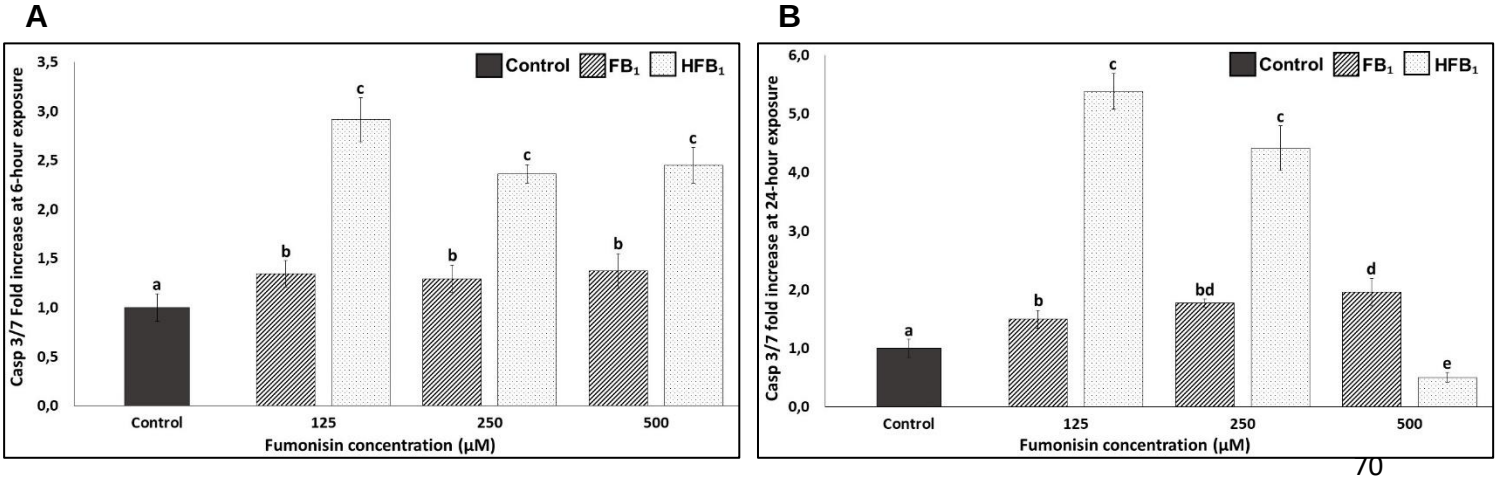


Figure 7: The effect of fumonisin B_1 (FB_1) and hydrolyzed fumonisin B_1 (HFB_1) on cell viability (ATP production) at 6- (A and C) and 24-hour (B and D) incubation periods in IPEC-J2 porcine intestinal cells. ATP- adenosine triphosphate production was calculated as a percentage shown in section 3.2.5.1. Statistical analyses were included, and statistical significance considered at $p < 0.05$. Concentrations include a control and various concentrations of FB_1 and HFB_1 (from 0.98 µM to 500 µM). Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration of FB_1 and HFB_1 are compared to the control.

The porcine intestinal cells were also subjected to FB_1 and HFB_1 exposure for 24 hours. As illustrated in Figure 7 (B and D) following this exposure, FB_1 elicited a significant ($p < 0.05$) reduction in cell viability at concentrations of 0.98 µM and 7.81 µM. In contrast, cell viability remained largely unaffected at all other concentrations when compared to the control group. The cell viability significantly decreased ($p < 0.05$) after exposure to HFB_1 for a 24-hour period at a concentration of 62.5 µM and lower. A significant increase ($p < 0.01$) was observed at 250 µM HFB_1 exposure (> 120 %), followed by a significant decline in cell viability at 500 µM exposure, with viability reduced to less than 40 % in comparison to the control group.

3.3.1.2 The effect of FB_1 and HFB_1 exposure on apoptosis

To determine the impact of FB_1 and HFB_1 on cell death, apoptosis was measured in the IPEC-J2 cells after exposure to FB_1 and HFB_1 for 6 and 24 hours. Following a 6-hour FB_1 exposure (Figure 8A and C), a significant increase ($p < 0.05$) in apoptosis (< 1-fold) was observed at concentrations of 125 µM and higher. All concentrations lower than 125 µM showed no significant differences when compared to the control. Exposure to HFB_1 after 6 hours exhibited a significant decline ($p < 0.05$) in apoptosis at concentrations 3.91 µM and below. Apoptosis at concentrations above 31.25 µM was induced and peaked at 125 µM, resulting in a significant 3-fold increase ($p < 0.05$) compared to the control. Concentrations surpassing 250 µM exhibited a plateau effect yet still elicited apoptotic responses greater than 2-fold.



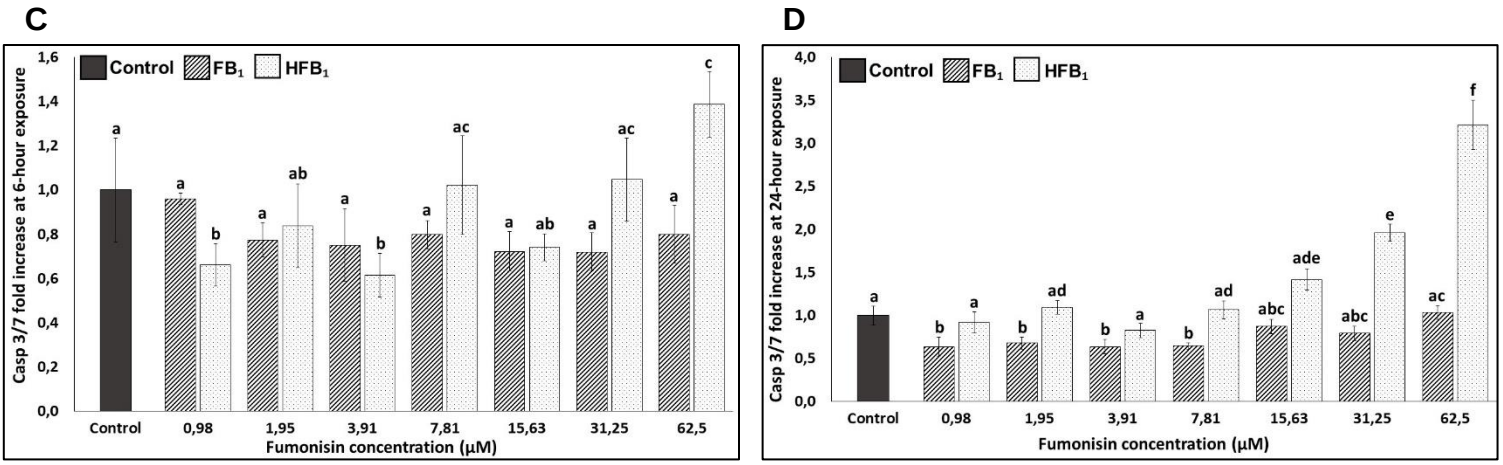


Figure 8: The effect of fumonisin B_1 (FB_1) and hydrolyzed fumonisin B_1 (HFB_1) on apoptosis (Caspase 3/ 7-fold increase) at 6- (A and C) and 24-hour (B and D) incubation periods in IPEC-J2 porcine intestinal cells. Apoptosis was calculated using Casp- Caspase 3/ 7-fold increase shown in section 3.2.5.2. Statistical analyses were included and statistical significance was considered at $p < 0.05$. Concentrations include a control and various concentrations of FB_1 and HFB_1 (from 0.98 µM to 500 µM). Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration of FB_1 and HFB_1 are compared to the control.

Subsequent to 24 hours FB_1 exposure (Figure 8B and D), concentrations from 0.98 µM exhibited a significant decrease ($p < 0.05$) in apoptotic events (± 0.5-fold). Apoptosis was then increased 2-fold in a dose-dependent manner at concentrations higher than 500 µM. Following 24-hour HFB_1 exposure within IPEC-J2 cells, apoptosis was induced dose-dependently from 15.63 µM (<1.5-fold) and peaked < 5-fold at 125 µM when compared to the control. Additionally, a significant decline ($p < 0.01$) in apoptosis followed at 500 µM HFB_1 exposure (> 1-fold).

3.3.1.3 The effect of FB_1 and HFB_1 exposure on cell proliferation

To assess the impact of FB_1 and HFB_1 on IPEC-J2 cell proliferation, cells were subjected to exposure to these fumonisins for durations of 6 and 24 hours. Following a 6-hour exposure to FB_1, a slight decrease ($p < 0.05$) (> 90 %) in cell proliferation was observed at concentrations 62.5 µM and below (Figure 9A and C). However, no cell proliferation was observed at FB_1 concentrations from 125 µM and higher compared to the control. After 6 hours HFB_1 exposure, concentrations exceeding 62.5 µM exhibited a substantial attenuation in cell proliferation, with a significant dose-dependent reduction ($p < 0.01$) exceeding 40 %.

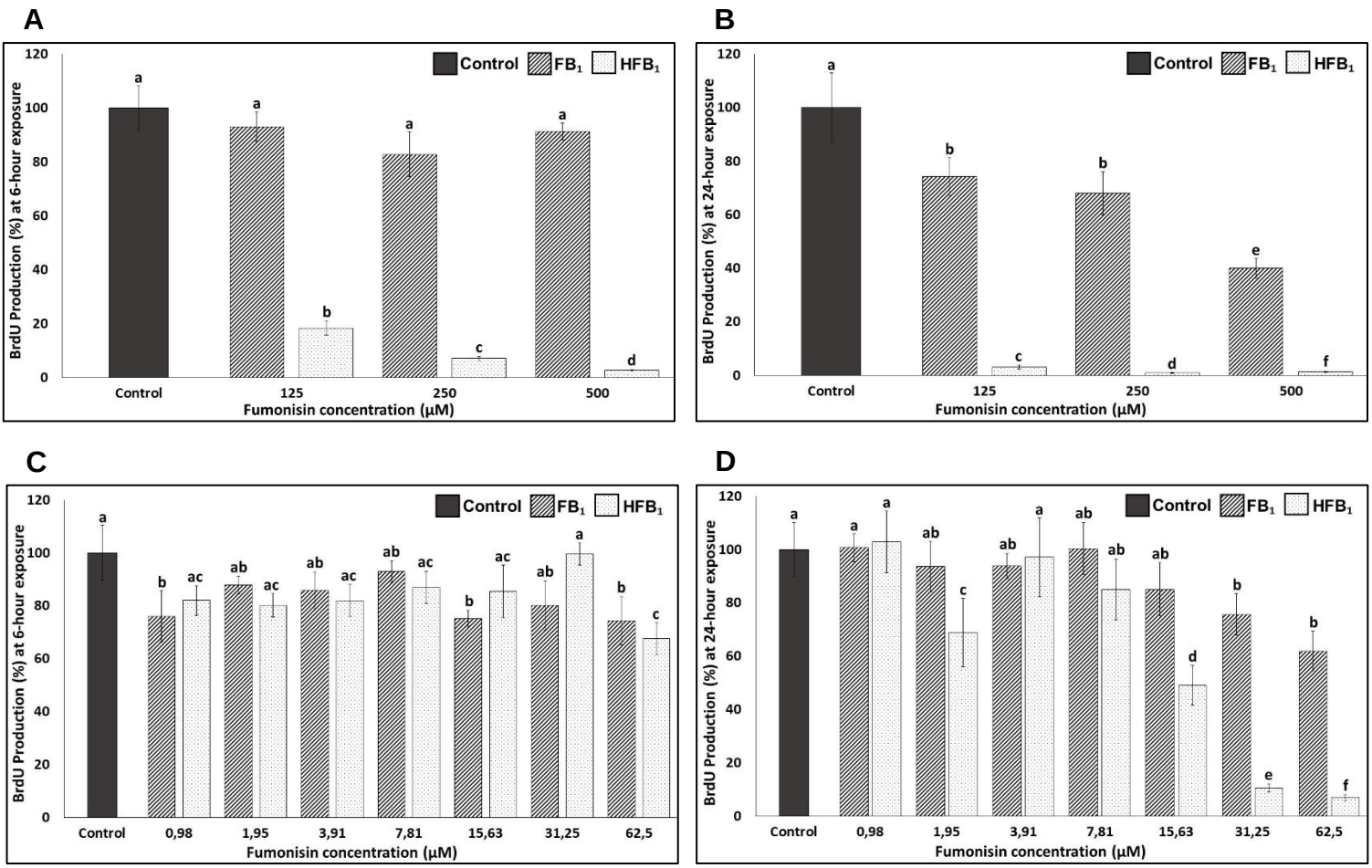


Figure 9: The effect of fumonisin B_1 (FB_1) and hydrolyzed fumonisin B_1 (HFB_1) on cell proliferation (BrdU production) at 6- (A and C) and 24-hour (B and D) incubation periods in IPEC-J2 porcine intestinal cells. BrdU- Bromodeoxyuridine was calculated and is shown as a percentage (BrdU calculations provided in section 3.2.5.3). Concentrations include a control and various concentrations of FB_1 and HFB_1 (from 0.98 µM to 500 µM). Statistical analyses were included and statistical significance was considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration of FB_1 and HFB_1 are compared to the control.

After 24 hours exposure to FB_1 (Figure 9B and D), cell proliferation decreased dose- dependently at concentrations from 1.95 to 500 µM respectively (from < 90 % to > 40 %). After 24 hours exposure to HFB_1, cell proliferation decreased dose-dependently from 7.81 µM and was significantly decreased ($p < 0.05$) at concentrations above 31.25 µM (> 10 %) compared to the control.

3.3.1.4 The effect of FB_1 and HFB_1 exposure on IL-8 concentration

The impact of IL- 8 concentration on IPEC-J2 cells was evaluated after exposure to FB_1 and HFB_1 for 6 and 24 hours. Following 6 hours exposure to FB_1 (Figure 10A and C), IL-8 concentration

was downregulated at concentrations 250 µM and above. However, no significant differences were observed at concentrations below 125 µM compared to the control. Subsequently, 6 hours HFB_1 exposure, a dose-dependent decrease in IL-8 was observed at concentrations from 15.63 to 500 µM respectively (from ± 250 pg/ mL to ± 100 pg/ mL) when compared to the control (± 270 pg/ mL).

Following 24 hours FB_1 exposure (Figure 10B and D), cells elicited a significant down-regulation ($p < 0.01$) of IL-8 concentration at 0.98 µM (> 600 pg/ mL) and increased dose-dependently up to 250 µM (< 1400 pg/ mL) when compared to the control. After 24 hours HFB_1 exposure, a significant dose-dependent down-regulation ($p < 0.01$) of IL-8 concentration was observed from 15.63 to 500 µM respectively (from > 1500 pg /mL to > 200 pg/ mL) when compared to the control (± 1000 pg/ mL).

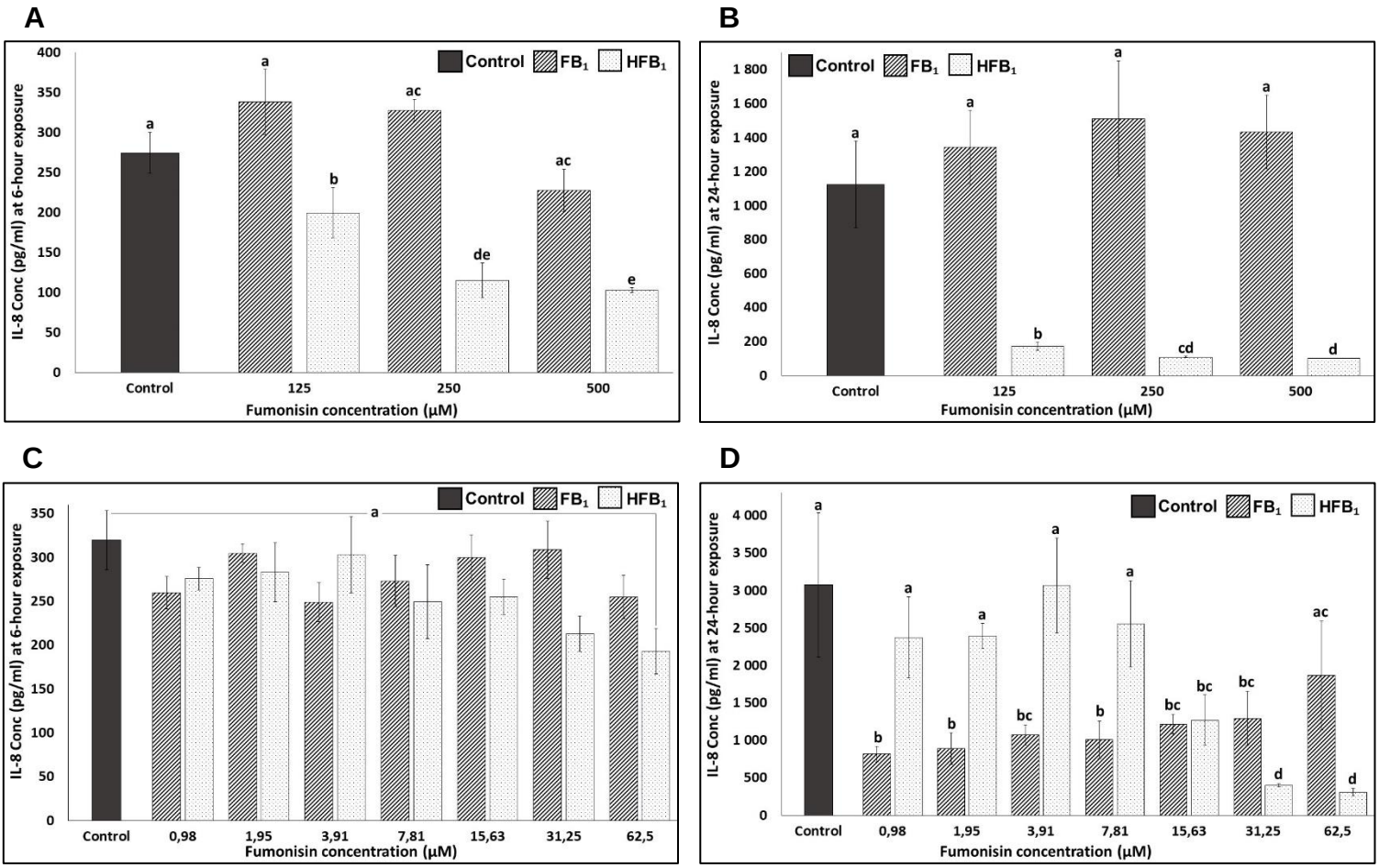


Figure 10: The effect of fumonisin B_1 (FB_1) and hydrolyzed fumonisin B_1 (HFB_1) on an inflammatory biomarker (IL-8: interleukin 8 Conc: concentration) at 6- (A and C) and 24-hour (B and D) incubation periods in IPEC-J2 porcine intestinal cells. (IL-8 was calculated as shown in section 3.2.5.4). Concentrations include a control and various concentrations of FB_1 and HFB_1 (from 0.98 µM to 500 µM). Statistical analyses were included and statistical significance was considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration of FB_1 and HFB_1 are compared to the control.

3.3.2 The effect of lipopolysaccharides exposure on IPEC-J2 cell viability indices

The impact of LPS on cell viability indices was determined by exposing IPEC-J2 cells to various concentrations of LPS (from 1.25 µg/ mL to 10 µg/ mL respectively) for 6 and 24 hours. The preparation and seeding calculations are provided in section 3.2.4. Cells were subjected to LPS exposure to observe the effects of cell viability, apoptosis, cell proliferation and IL-8 concentration depicted in Figure 11, 12, 13 and 14 respectively.

3.3.2.1 The effect of LPS exposure on cell viability

To determine the effect of LPS on cell viability, cells were exposed to LPS for 6 and 24 hours. Following 6 hours exposure to LPS (Figure 11A), a slight decrease ($p < 0.01$) in cell viability was observed at 2.5 µg/ mL (> 90 %) compared to the control. A similar trend was observed after 24 hours exposure with LPS (Figure 11B).

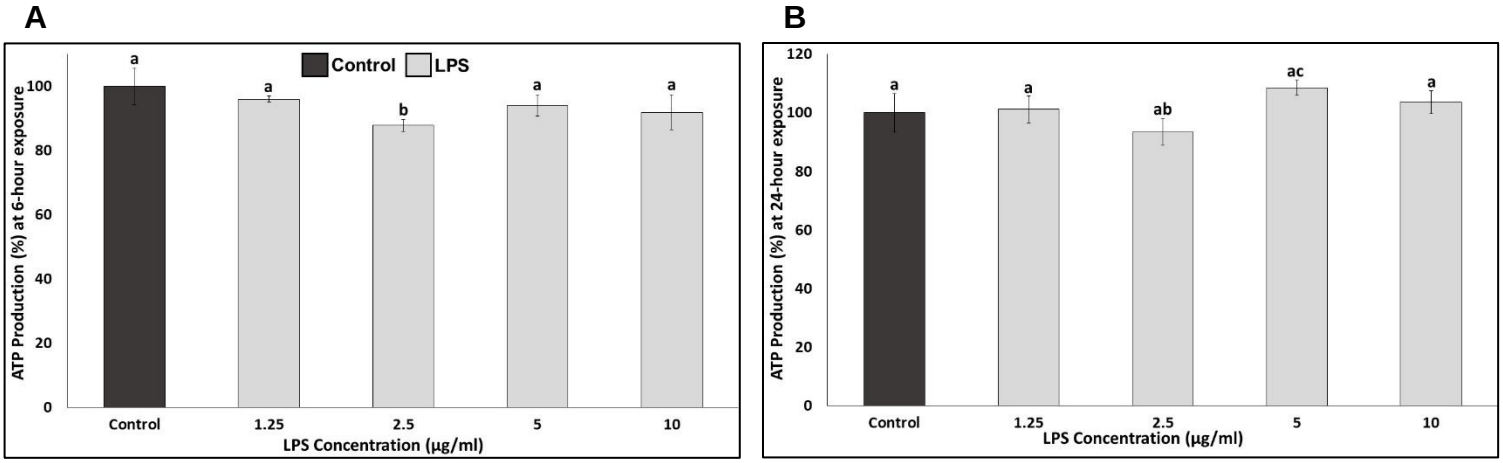


Figure 11: The effect of lipopolysaccharides (LPS) on cell viability (ATP production), at 6- (A) and 24-hour (B) incubation periods in IPEC-J2 porcine intestinal cells. ATP- adenosine triphosphate production was calculated as a percentage shown in section 3.2.5.1. Concentrations include a control and various concentrations of LPS (from 1.25 µg/ mL to 10 µg/ mL). Statistical analyses were included and statistical significance was considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration of LPS is compared to the control.

3.3.2.2 The effect of LPS exposure on apoptosis

The effect of LPS on apoptosis was determined after cells were exposed to LPS for 6 and 24 hours. No significant differences were observed following 6 hours exposure to LPS (Figure 12A). However, apoptosis was significantly induced ($p < 0.01$) at concentrations 1.25 µg/ mL and 10 µg/ mL (< 1.5-fold) after 24 hours LPS exposure (Figure 12B), when compared to the control group.

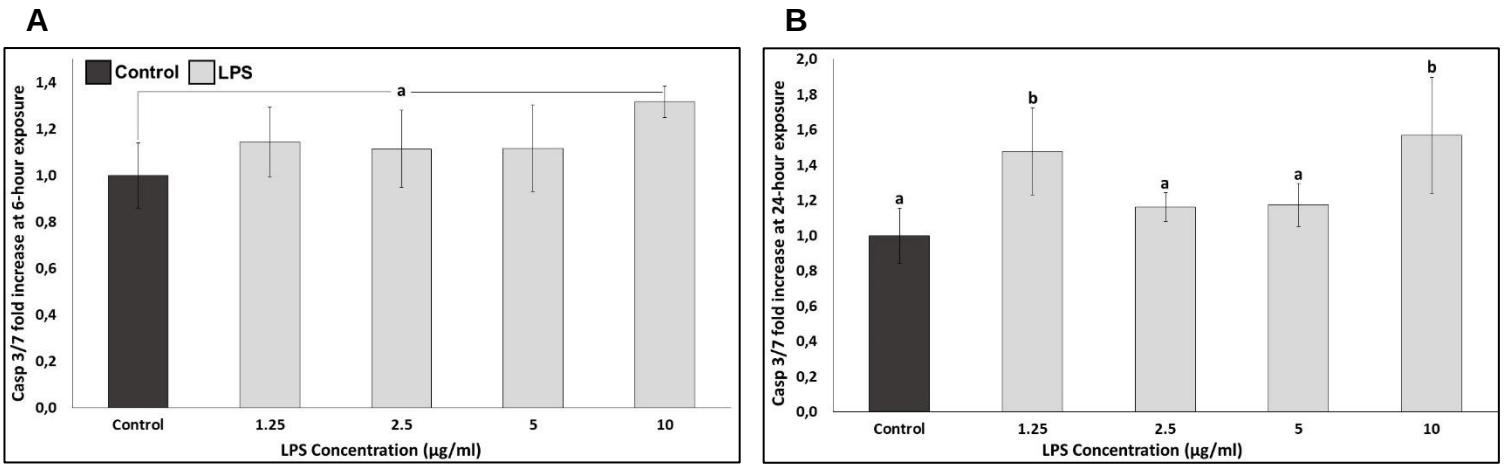


Figure 12: The effect of lipopolysaccharides (LPS) on apoptosis (Caspase 3/ 7-fold increase) at 6- (A) and 24-hour (B) incubation periods in IPEC-J2 porcine intestinal cells. Apoptosis was calculated using Casp- Caspase 3/ 7-fold increase shown in section 3.2.5.2. Concentrations include a control and various concentrations of LPS (from 1.25 µg/ mL to 10 µg/ mL). Statistical analyses were included and statistical significance considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration of LPS is compared to the control.

3.3.2.3 The effect of LPS exposure on cell proliferation

The impact of LPS on cell proliferation was determined after cells were exposed to LPS for 6 and 24 hours. No significant differences were observed at any of the LPS concentrations despite a significant decrease ($p < 0.05$) in cell proliferation (> 80 %) at 2.5 µg/ mL following 6 hours LPS exposure (Figure 13A and B).

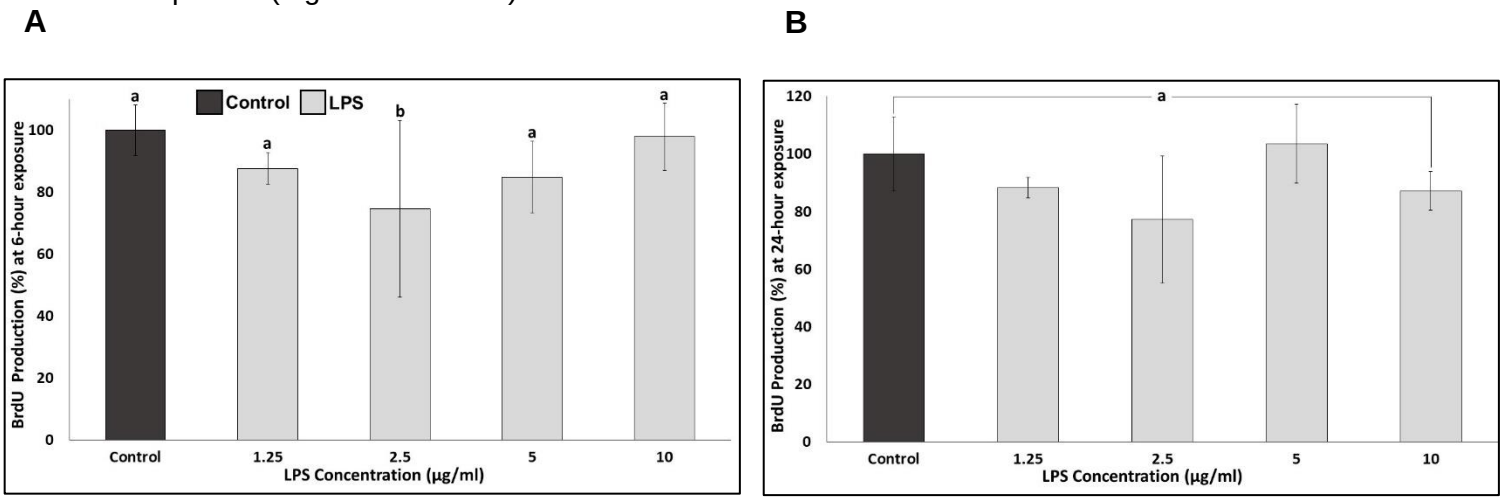


Figure 13: The effect of lipopolysaccharides (LPS) on cell proliferation (BrdU production) at 6- (A) and 24-hour (B) incubation periods in IPEC-J2 porcine intestinal cells. BrdU- Bromodeoxyuridine was calculated and is shown as a percentage (BrdU calculations provided in section 3.2.5.3). Concentrations include a control and various concentrations of LPS (from 1.25 µg/ mL to 10 µg /mL). Statistical analyses were included and statistical significance was considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration of LPS is compared to the control.

3.3.2.4 The effect of LPS exposure on IL-8 concentration

The impact of LPS on IL-8 concentration was determined subsequent to 6 and 24 hours LPS exposure. It was observed that IL-8 concentration was significantly induced ($p < 0.05$) in a dose-dependent manner compared to the control (> 300 pg/ mL), from 1.25 µg/ mL (< 500 pg/ mL) following 6 hours LPS exposure (Figure 3.8A). IL-8 concentration was also significantly increased ($p < 0.05$) at concentrations 2.5 µg/ mL and 10 µg/ mL (< 1500 pg/ mL) following 24 hours LPS exposure (Figure 14B).

A **B**

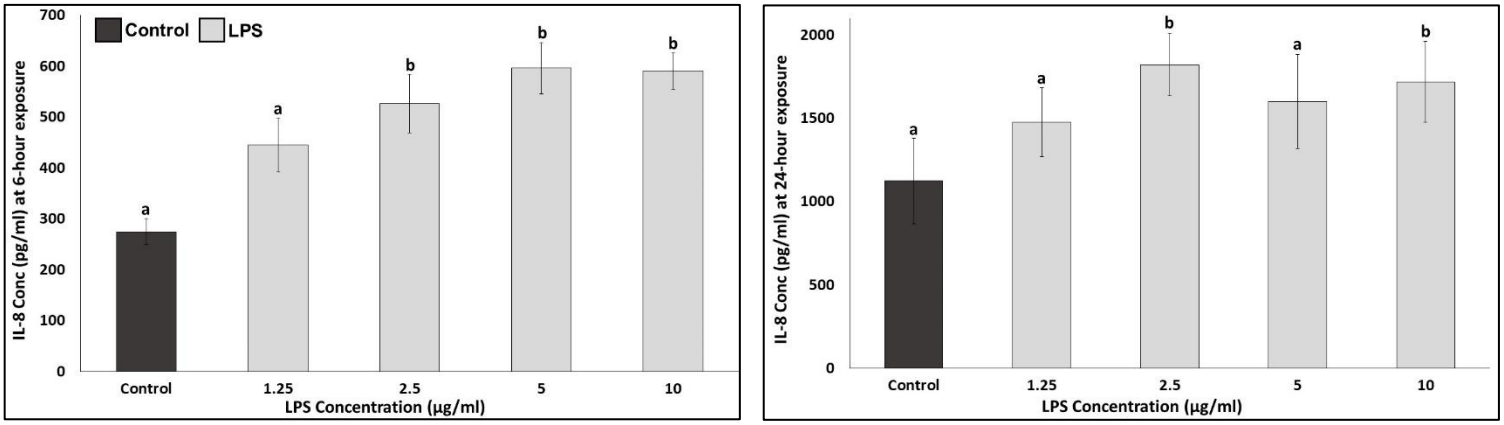


Figure 14: The effect of lipopolysaccharides (LPS) on an inflammatory biomarker (IL-8 Concentration) at 6- (A) and 24-hour (B) incubation periods in IPEC-J2 porcine intestinal cells. (IL-8: Interleukin 8 Conc: concentration was calculated as shown in section 3.2.5.4). Concentrations include a control and various concentrations of LPS (from 1.25 µg/ mL to 10 µg/ mL). Statistical analyses were included and statistical significance was considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration of LPS is compared to the control.

3.3.3 The co-exposure effect of fumonisin B_1, hydrolyzed fumonisin B_1 and lipopolysaccharides on IPEC-J2 cell survival indices

The co-exposure effect of FB_1 and HFB_1 with LPS was determined after cells were exposed to two concentrations of FB_1 and HFB_1, and one concentration of LPS individually for 24 hours. Cells were also exposed to a co-exposure treatment of previously chosen concentrations that are provided in section 3.2.2.2 for the same exposure period to determine the effects of cell viability, apoptosis, cell proliferation and IL-8 concentration depicted in Figure 15, 16, 17 and 18 respectively.

3.3.3.1 The effect of FB_1, HFB_1 and LPS on cell viability

The impact of FB_1, HFB_1, and LPS on cell viability was determined after exposing IPEC-J2 cells to these fumonisins and endotoxin for 24 hours (Figure 15). A significant decrease ($p < 0.05$) in cell viability was observed at 10 µg/ mL LPS and non-significantly with 15.63 µM FB_1 exposure (> 90 %) when compared to the control. Additionally, a slight increase in cell viability was noted at individual 15.63 µM HFB_1 and FB_1 + HFB_1 co-exposure (< 100 %).

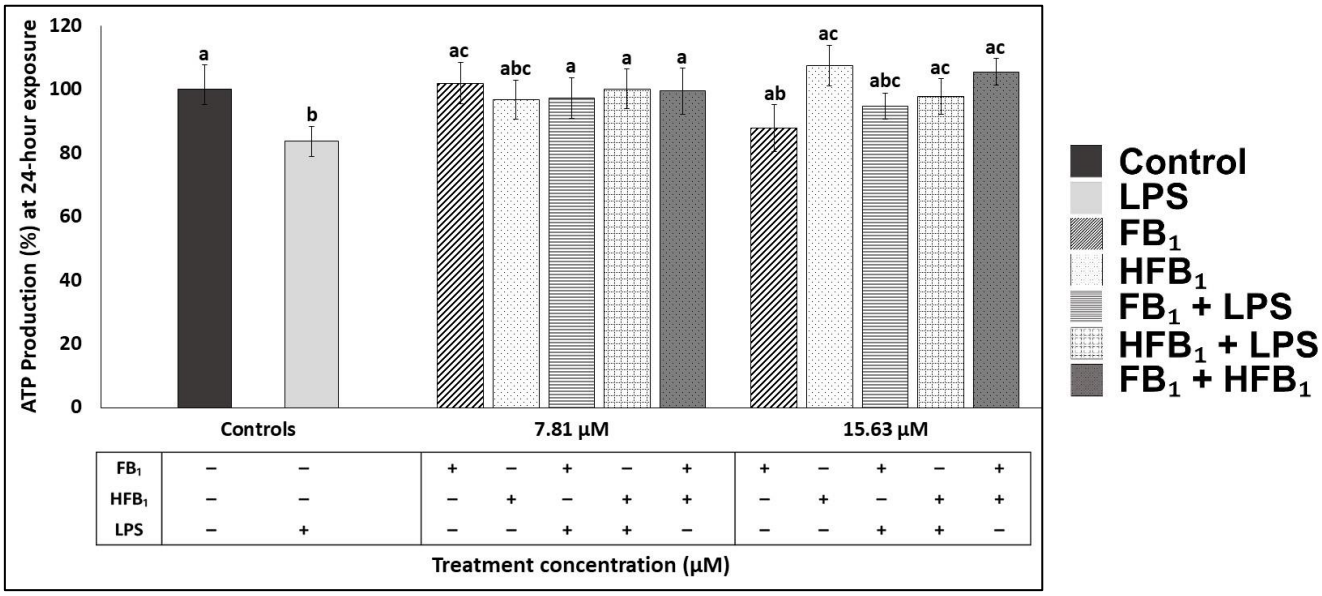


Figure 15: The effect of fumonisin B_1 (FB_1), hydrolyzed fumonisin B_1 (HFB_1) and lipopolysaccharides (LPS) on cell viability (ATP Production) after 24 hours exposure in IPEC-J2 porcine intestinal cells. ATP-adenosine triphosphate production was calculated as a percentage shown in section 3.2.5.1. Concentrations include the control, 10 µg/ mL LPS (LPS), 7.81 µM and 15.63 µM FB_1, HFB_1, FB_1 in the presence of 10 µg/ mL LPS (FB_1 + LPS), HFB_1 in the presence of 10 µg/ mL LPS (HFB_1 + LPS) and FB_1 in the presence of HFB_1 (FB_1 + HFB_1). Statistical analyses were included and statistical significance was considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration is compared to the control.

3.3.3.2 The effect of FB_1, HFB_1 and LPS on apoptosis

The combined effect of FB_1, HFB_1, and LPS on apoptosis were determined by exposing cells to these toxins for 24 hours (Figure 16). Following individual and co-exposure, a significant decrease ($p < 0.05$) in apoptosis was observed at 7.81 µM FB_1, 7.81 µM HFB_1 + LPS co-exposure, and 7.81 µM FB_1 + HFB_1 co-exposure (> 0.8-fold) when compared to the control. Additionally, 15.63 µM FB_1 and HFB_1, as well as 15.63 µM FB_1 + HFB_1 co-exposure induced a larger apoptotic response when compared to their respective 7.81 µM concentrations. Another significant

observation showed that HFB_1 + LPS co-exposure decreased apoptosis when compared to the respective individual HFB_1 exposure.

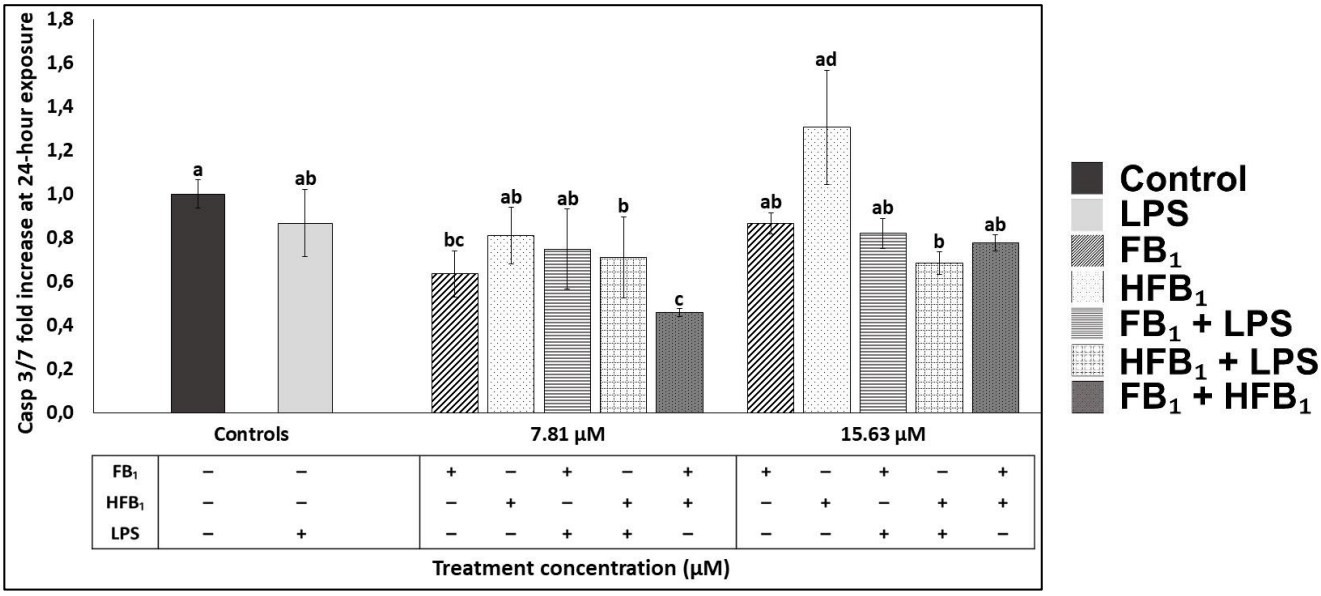


Figure 16: The effect of fumonisin B_1 (FB_1), hydrolyzed fumonisin B_1 (HFB_1) and lipopolysaccharides (LPS) on apoptosis (Caspase 3/7-fold) after 24 hours exposure in IPEC-J2 porcine intestinal cells. Apoptosis was calculated using Casp- Caspase 3/7-fold increase shown in section 3.2.5.2. Concentrations include the control, 10 µg/ mL LPS (LPS), 7.81 µM and 15.63 µM FB_1, HFB_1, FB_1 in the presence of 10 µg/ mL LPS (FB_1 + LPS), HFB_1 in the presence of 10 µg/ mL LPS (HFB_1 + LPS) and FB_1 in the presence of HFB_1 (FB_1 + HFB_1). Statistical analyses were included and statistical significance was considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration is compared to the control.

3.3.3.3 The effect of FB_1, HFB_1 and LPS on cell proliferation

The combined effect of FB_1, HFB_1 and LPS exposure on cell proliferation was determined after exposing cells to these toxins for 24 hours (Figure 17). Following individual and co-exposure, a significant increase ($p < 0.05$) in cell proliferation occurred with 10 µg/ mL LPS, 15.63 µM FB_1, and 15.63 µM FB_1 + LPS co-exposure (< 120 %) when compared to the control (100 %). A slight decrease in cell proliferation was observed at 15.63 µM HFB_1 and a significant decrease ($p < 0.05$) at 15.63 µM FB_1 + HFB_1 co-exposure (> 90 %) when compared to the control (100 %).

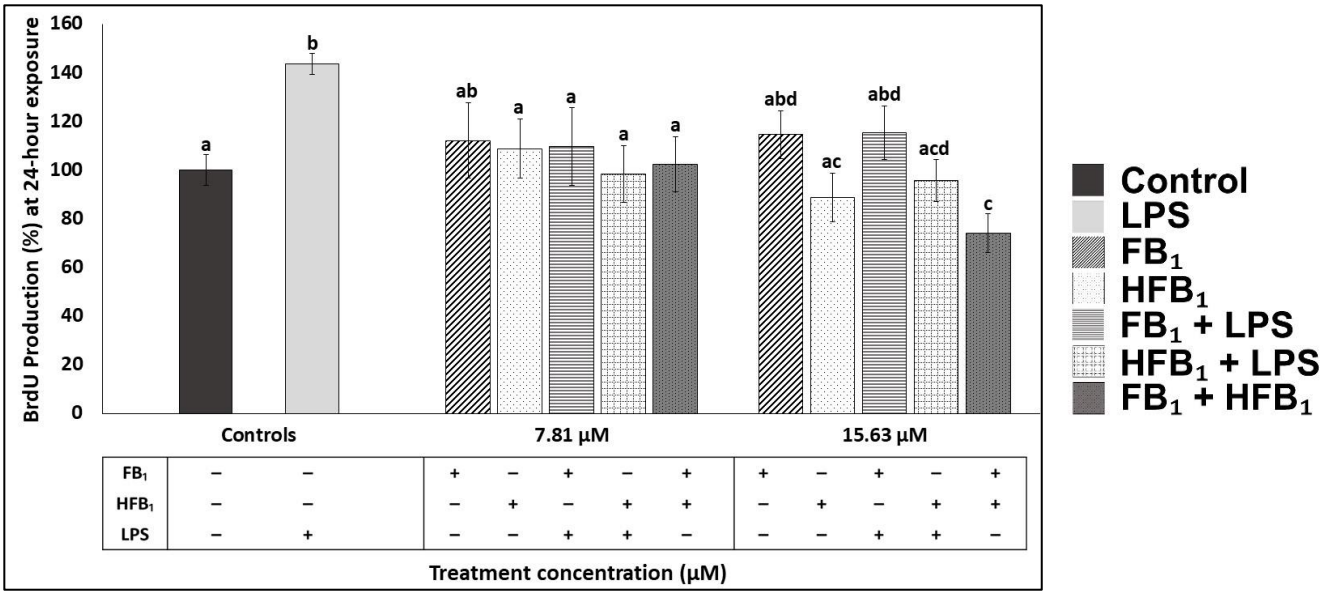


Figure 17: The effect of fumonisin B_1 (FB_1), hydrolyzed fumonisin B_1 (HFB_1) and lipopolysaccharides (LPS) on cell proliferation (BrdU Production) after 24 hours exposure in IPEC-J2 porcine intestinal cells. BrdU-Bromodeoxyuridine was calculated and is shown as a percentage (BrdU calculations provided in section 3.2.5.3). Concentrations include the control, 10 µg/ mL LPS (LPS), 7.81 µM and 15.63 µM FB_1, HFB_1, FB_1 in the presence of 10 µg/ mL LPS (FB_1 + LPS), HFB_1 in the presence of 10 µg/ mL LPS (HFB_1 + LPS) and FB_1 in the presence of HFB_1 (FB_1 + HFB_1). Statistical analyses were included and statistical significance was considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration is compared to the control.

3.3.3.4 The effect of FB_1, HFB_1 and LPS on IL-8 concentration

The combined effect of FB_1, HFB_1 and LPS on IL-8 concentration was determined after exposing IPEC-J2 cells to these fumonisins and LPS for 24 hours (Figure 18). Following LPS exposure, cells elicited a slight induction of IL-8 concentration (< 3500 pg/ mL) when compared to the control (> 3500 pg/ mL). Individually, FB_1 and HFB_1 exposure at their respective 7.81 and 15.63 µM concentrations produced similar effects, i.e. no effect with FB_1, whereas HFB_1 significantly decreased ($p < 0.01$) IL-8. In the presence of LPS, both FB_1 and HFB_1 (not significant) concentrations produced an up-regulation of IL-8 concentration (< 3500 pg/ mL and < 2500 pg/ mL respectively) compared to the non-LPS treated. FB_1 + HFB_1 co-exposure significantly down-regulated ($p < 0.01$) IL-8 concentration at both 7.81 µM and 15.63 µM concentrations when compared to the control.

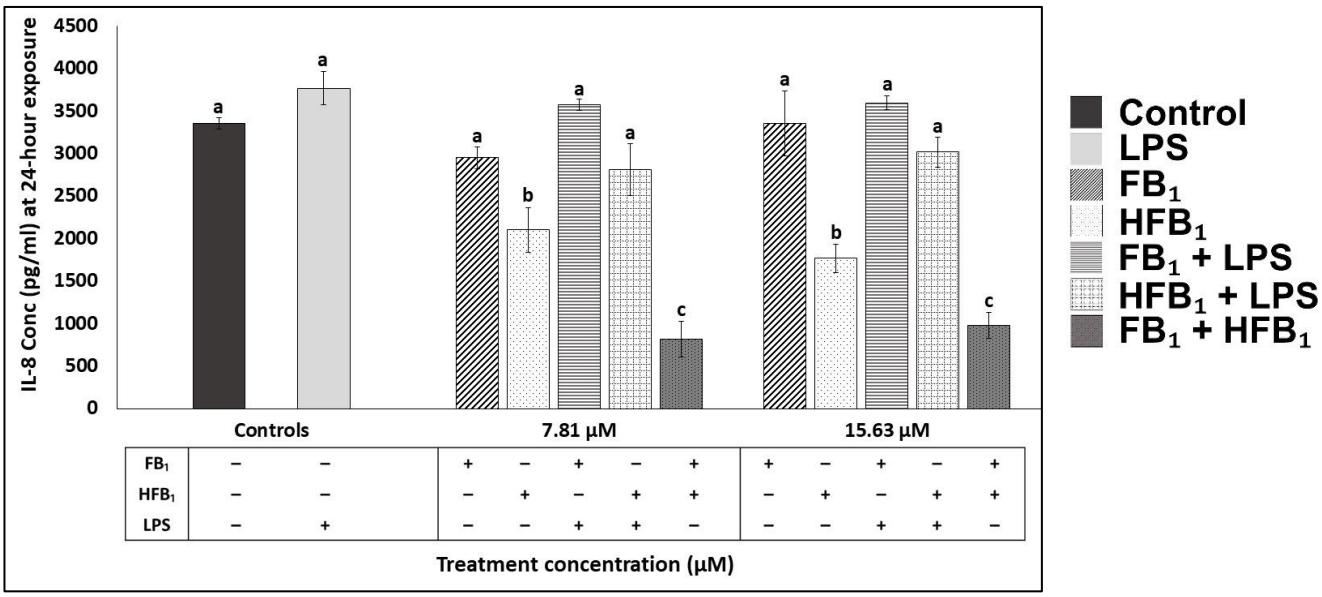


Figure 18: The effect of fumonisin B_1 (FB_1), hydrolyzed fumonisin B_1 (HFB_1) and lipopolysaccharides (LPS) on an inflammatory biomarker (IL-8 Concentration) after 24 hours exposure in IPEC-J2 porcine intestinal cells. (IL-8: Interleukin 8 Conc: concentration was calculated as shown in section 3.2.5.4). Concentrations include the control, 10 µg/ mL LPS (LPS), 7.81 µM and 15.63 µM FB_1, HFB_1, FB_1 in the presence of 10 µg/ mL LPS (FB_1 + LPS), HFB_1 in the presence of 10 µg/ mL LPS (HFB_1 + LPS) and FB_1 in the presence of HFB_1 (FB_1 + HFB_1). Statistical analyses were included and statistical significance was considered at $p < 0.05$. Statistically significant differences are shown as lowercase letters above each of the graph bars where each concentration is compared to the control.

3.4 Discussion

3.4.1 The effect of fumonisin B_1 on intestinal epithelial cells

The gastrointestinal tract represents the first barrier against the ingestion of various dietary substances (Szabó *et al.*, 2023). Intestinal epithelial cells regulate the intestinal barrier function and are constantly being exposed to large quantities of contaminants and toxins such as FB_1 and HFB_1 (Bouhet and Oswald, 2007) with subsequent negative health effects. In this study, we optimized the IPEC-J2 cell line by exposing it to various concentrations of FB_1, HFB_1 and LPS individually and subsequently within a co-exposure model to determine effects on cellular intestinal viability.

FB_1 contamination in pigs induces toxic effects in organs such as the lungs, liver and kidneys (Haschek *et al.*, 2001). It was also shown to cause intestinal barrier disruption through the disturbance of tight junction function (Holanda and Kim, 2021). Studies performed by Chen *et al.* (2019) and Wan *et al.* (2013) showed that IPEC-J2 cells exposed to FB_1 for 48 hours experienced a significant decline in cell viability at concentrations above 25 µM and 40 µM, respectively. Other intestinal cell lines such as the porcine iliac artery endothelial cells were also sensitive to FB_1 exposure and resulted in a significant decrease in cell viability after 48 hours 50 µg/ mL FB_1 exposure (Yuan *et al.*, 2019). However, 24 hours exposure to various concentrations (1 µM - 70 µM) of FB_1 did not affect cell viability in HT-29 cells, and only exhibited a slight decline after 72 hours 70 µM FB_1 exposure (Minervini *et al.*, 2014). Similarly, the current study showed that FB_1 concentrations up to 500 µM exposure produced no significant effects on cell viability in the IPEC-J2 cells after 24 hours. Many of the above studies showed that there was no effect on cell viability after 24 hours FB_1 exposure. This suggests that the effect of FB_1 on intestinal cells becomes more cytotoxic dose-dependent as time progresses, similar to the findings stated by Wang *et al.* (2022).

Many previous studies report that FB_1 induces apoptosis in various cell lines. It was shown to induce apoptosis through the induction of caspase-3 and apoptotic proteins in rat liver cells, TNF-α signalling pathways in pig kidney cells, and stimulate the proliferation of human endometrial endothelial cells (Cao *et al.*, 2022, Chen *et al.*, 2020, Wang *et al.*, 2020, Wang *et al.*, 2014). In the current study, IPEC-J2 cells presented an increased apoptotic response at concentrations above 125 µM FB_1 following 6- and 24-hour exposure periods. This suggests that cells were under stress from 125 µM exposure independent of the exposure period. Wang *et al.* (2022) observed a similar finding in IPEC-J2 cells that displayed caspase- 3 and -9 cleavage and expression after 48 hours from 10 µM up to 40 µg/ mL FB_1 exposure.

Additionally, it is noted that cells may seem viable when performing cell viability assays. However, upon performing additional assays to determine the cell survival indices (such as cell proliferation assays), a significant decrease would be observed at levels of sufficient viability. It was reported that after 48 hours exposure to FB_1 concentrations of 25 μg/ mL and above, induces injury on pig epithelial cells by inhibiting cell proliferation (Chen *et al.*, 2019). IPEC-J2 cells exposed to 40 μM FB_1 exhibited inhibition of cell proliferation dose-dependent through the inhibition of i) G1/ S phase gene expression, ii) promotion of mRNA expression of cycle suppression genes, and iii) blockage of cell cycle G1 phase. HT-29 cells exposed to FB_1 concentrations of 8.6 μM and higher also elicited a dose-dependent inhibition of cell proliferation after 72 hours of exposure (Minervini *et al.*, 2014). A similar trend was observed in the current study as concentrations above 31.25 μM FB_1 exposure promoted a dose-dependent cell proliferation inhibition after 24 hours.

One of the cytokines responsible for enhancing cell proliferation, promoting inflammation and controlling the repair processes during intestinal mucosal injury or cytotoxic stress is the activity of IL-8. A study performed using IPEC-1 cells observed a dose-dependent decrease in IL-8 concentration at mRNA and B-protein level after 4 days up to 72.2 μM FB_1 exposure (Bouhet *et al.*, 2006). Another study observed no IL-8 response on HT-29 cells after 48-hour FB_1 exposure (Minervini *et al.*, 2014). The current study shows a dose-dependent induction of IL-8 at all concentrations up to 31.25 μM FB_1 exposure, which was also observed following 125 μM FB_1 exposure after 24 hours. This result coupled with cell proliferation depicts that IL-8 was secreted upon FB_1 exposure beyond 31.25 μM, indicative of an inflammatory response while also making it dose and time dependent.

3.4.2 The effect of hydrolyzed fumonisin B_1 on intestinal epithelial cells

There are many studies that demonstrate that hydrolyzed fumonisins are less toxic than fumonisins (Grenier *et al.*, 2012, Gu *et al.*, 2019). Many mammalian *in vivo* studies have supported these findings (Grenier *et al.*, 2012, Hahn *et al.*, 2015, Howard *et al.*, 2002). Voss *et al.* (2009) showed that ingesting FB_1-contaminated feed promoted neural tube defects, increased fetal death rates and decreased fetal weights in dams compared to HFB_1 administration, which did not cause any adverse effects besides a slight disruption of the sphingolipid metabolism. Another study found HFB_1 to be 100–fold less toxic than FB_1 in cultured rat embryos (Flynn *et al.*, 1997). Furthermore, a study performed by Gelderblom *et al.* (1993) used rat liver cancer initiation with dietary fumonisins where HFB_1 failed to initiate cancer. However, it was found to be more cytotoxic than the parent molecule in culture, which suggests that HFB_1 could become more cytotoxic under

certain conditions (Gelderblom *et al.*, 1993). Additionally, when HFB_1 is in the presence of palmitoyl-CoA, it becomes acylated and forms N-palmitoyl HFB_1 which was 10-fold more toxic than FB_1 in an *in vitro* mammalian test model (Abou-Karam *et al.*, 2004). There are few mammalian *in vitro* models exploring the comparison toxicological effects of HFB_1 to FB_1, which makes this area of research controversial (Abbax *et al.*, 1993, Caloni *et al.*, 2002, Dombrink-Kurtzman, 2003). It is suggested that the toxic effects of HFB_1 within mammalian *in vitro* study models may be further described through the use of extended exposure periods with exceedingly high HFB_1 concentrations which cannot be obtained through dietary exposure.

In the current study, the increase in cell viability after 250 µM HFB_1 exposure, followed by a rapid decline at 500 µM exposure, was observed and indicative of cytotoxicity. The increase in cell viability at particular concentrations relates to a phenomenon that stressed or dying cells release or expose molecules called damage-associated molecular patterns (DAMPs), such as ATP and amphotericin on their surface which signals the innate immune system, and increases inflammation. Extracellular ATP specifically is often released from apoptotic cells where the secretion is associated with the apoptotic stage and type of stress or cell death stimulus (Krysko *et al.*, 2012). This suggests that cells exhibiting an initial increase followed by a rapid decline in cell viability is indicative of cellular stress.

It was expected that cells would undergo apoptosis after 24 hours following 125 µM HFB_1 exposure due to the decrease observed in cell viability at that particular point. However, it was observed that after 6 hours HFB_1 exposure, cells induced apoptosis following 62.5 µM HFB_1 exposure. The apoptotic response after 24 hours HFB_1 exposure exceeded that of 6 hours. Cell proliferation further indicated that there were very few cells proliferating above 62.5 µM HFB_1 exposure after 6 hours. This suggests that, like FB_1, prolonged exposure of HFB_1 promotes dose and time-dependent cytotoxicity. The rapid decrease in apoptosis at concentrations following 250 µM HFB_1 could be due to the lack of cells available in the late stages of apoptosis. Another reason for this decline could be the caspase assay itself, as it was performed on the cell lysate, which does not contain the dead cells present in the supernatant; which could result in a smaller percentage of cells within wells as well as lower apoptosis values. Additionally, cells may also be undergoing a rapid onset of cellular necrosis after a high concentration of HFB_1 exposure and may not have enough time or energy to initiate apoptotic mechanisms which will therefore not express apoptotic indicators, resulting in lowered apoptosis (Istifli *et al.*, 2019).

An *in vivo* study performed by Grenier *et al.* (2012) fed HFB_1-containing feed to pigs for 14 days and found that the proximal section of the intestine (where IPEC-J2 cells stem from) produced a higher concentration of IL-8 compared to FB_1. In the current study, a similar trend was observed at concentrations up to 7.81 µM HFB_1 after 24 hours exposure. However, an anti-inflammatory effect was observed following 125 µM and 15.63 µM HFB_1 exposure after 6 and 24 hours respectively. The decrease in IL-8 correlates with a decrease in cell viability and cell proliferation, and an increase in apoptosis. However, an increase in IL-8 promotes a decrease in the cell survival rate (Qazi *et al.*, 2011). This suggests that HFB_1 might be metabolized differently in the gut *in vivo* compared to *in vitro* and therefore promote the anti-inflammatory effect during cytotoxicity exposure.

3.4.3 The effect of lipopolysaccharides on intestinal epithelial cells

Lipopolysaccharide (LPS) is a potent inflammatory response inducer and may cause the immune system to become more susceptible to disease (Mazgaeen and Gurung, 2020). Therefore, LPS exposure triggers inflammatory responses within intestinal epithelial cells by activating and releasing various pro-inflammatory factors. A study performed by Zhao *et al.* (2020) demonstrated that IPEC-J2 cells exposed to 10 µg/ mL LPS for 24 hours, elicited a significant decrease in cell viability, and a significant increase in IL-8 and apoptosis compared to the control. In the current study, the identification of the optimum LPS concentration was needed to induce the largest inflammatory response and was then incorporated into the co-exposure model. Our data presented no significant decrease for cell viability following 24 hours LPS exposure. However, at 2.5 µg/ mL LPS exposure, a significant decrease in cell viability and cell proliferation was observed after 6 hours. Additionally, a significant increase in apoptosis at 1.25 µg/ mL and 10 µg/ mL concentrations after 24 hours LPS exposure was observed. This demonstrates that cellular stress occurred which then initiated the induction of pro-inflammatory cytokines as seen in the dose-dependent increase of IL-8 concentrations after 6 hours exposure. Moreover, a significant increase in IL-8 after 24 hours exposure to LPS concentrations of 2.5 µg/ mL and 10 ug/ mL respectively was also noted. It was evident that 10 ug/ mL LPS induced the greatest inflammatory response compared to the lower concentrations and was therefore incorporated into the co-exposure model with FB_1 and HFB_1.

3.4.4 The effect of fumonisin B_1, hydrolyzed fumonisin B_1 and lipopolysaccharide within a co-exposure model on intestinal epithelial cells

The identification of individual toxicity effects from various compounds creates a threshold when observing the effect of single doses on individual organisms. However, the combined effect of multiple organism toxicity from mycotoxins, bacteria or viruses may provide extensive information regarding toxicological interactions. Concurrent exposure to various toxins may induce synergistic, antagonistic or additive toxicity effects upon humans or animals (Alassane-Kpembi *et al.*, 2017). In the current study, LPS was used to induce inflammation within the IPEC-J2 cells upon FB_1 and HFB_1 exposure. This method was pursued to mimic the effect of fumonisin contamination occurring with preexisting inflammation in the gut. The addition of the FB_1 and HFB_1 was included to observe whether FB_1 toxicity will be reduced when adding a known less toxic substance such as HFB_1. The individual concentrations of FB_1, HFB_1 and LPS were analysed individually as it is required to observe the individual toxicity of mycotoxins as a base, prior to the effects of combination/ co-exposure toxicity (Yu *et al.*, 2023). The ideal FB_1 and HFB_1 concentrations (7.81 µM and 15.63 µM) used in the co-exposure model were obtained by choosing concentrations that did not elicit increased cell death or inflammation upon the IPEC-J2 cells after 24 hours exposure. The LPS concentration (10 µg/ mL) however, was chosen dependent on the highest level of inflammation induced in these cells. The exposure period was extended to 24 hours as no significant effects were observed after 6 hours exposure with FB_1, HFB_1 or LPS.

Individually and concurrent with LPS exposure, FB_1 exposed cells presented no significant effects on cell viability, cell proliferation and IL-8 concentration after 24 hours. Compared to a study that exposed macrophages to 0-50 µM FB_1 for 48 hours, exposure to LPS resulted in a slight decrease in cell viability. The duration of the exposure period could be the reason why no results were observed as some studies only obtained results after 48 hours of mycotoxin exposure (Dresden-Osborne and Noblet, 2002).

Individual 15.63 µM HFB_1 exposure presented an increase in apoptosis, and a decrease in cell proliferation and IL-8 concentration, similar to the individual concentrations observed above. However, combined with LPS, HFB_1 exposed cells promoted a slight increase in cell viability and IL-8 concentration, and a decrease in apoptosis, promoting an increased toxicity with the addition of LPS. Decreased apoptosis promotes an increased cell-survival rate which is often associated with the development of cancers (Gerl and Vaux, 2005). Although FB_1 was shown to induce

cancer in rat liver, intestinal cells exposed to HFB_1 may be prone to the proliferation of cancer when in the presence of inflammation (Gelderblom *et al.*, 1996b). This is coupled with a slight increase in cell proliferation in the presence of LPS at 15.63 µM HFB_1 exposure.

The combination/co-exposure of FB_1 with HFB_1 promoted a similar trend to that of the individual HFB_1 concentrations but to a higher degree. This suggests that HFB_1 effects may dominant between the two mycotoxins and be more cytotoxic in combination with FB_1, as FB_1 did not promote any adverse effects on the intestinal cells individually and with LPS exposure. However, the combination presented a greater adverse effect on the intestinal epithelial cells compared to the individual exposures. It appeared that the co-exposure had an anti-inflammatory effect, i.e., a decrease in IL-8, without having a detrimental effect on cell viability. This is in contrast to the individual effects of FB_1 and HFB_1 with LPS. This effect from the co-exposure can be mainly attributed to HFB_1.

Individually, it is noted that HFB_1 promotes greater toxicity in intestinal epithelial cells *in vitro* compared to FB_1. Although HFB_1 is known to be less toxic compared to FB_1 *in vivo*, which suggests that HFB_1 is metabolized differently *in vitro* compared to *in vivo*. This concept has to be further investigated to fully understand how FB_1 and HFB_1 interact with the gut. The data on mycotoxin co-exposure are limited and cannot always be predicted (Smith *et al.*, 2016). Likewise, the co-exposure of FB_1 with HFB_1 in this study are only indicative and would need to be further studied to determine the intricate interactive properties.

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CHAPTER 4: Comparative Proteomic Analysis Unveils Differential Responses to FB_1 and HFB_1 in Porcine IPEC-J2 Cell Line

ABSTRACT

The intestinal epithelium is constantly exposed to ingested harmful contaminants, such as mycotoxins. This can often lead to various forms of infection, which trigger stress response pathways, promoting delamination of cells and increased apoptosis. Fumonisin B_1 (FB_1) is a low molecular weight secondary metabolite that causes mycotoxicosis in various mammalian species. FB_1 can also be enzymatically converted to form hydrolyzed fumonisin B_1 (HFB_1), a less potent inhibitor of ceramide synthase, which was found to have controversial results in various *in vitro* and *in vivo* models. Although many studies focused on FB_1 and HFB_1 research individually, not many have identified the *in vitro* comparative mechanisms behind the variation of interactions at proteomics level. The identification of various signaling pathways provided after protein analysis, contributes to the increased understanding of the mechanisms behind pathogenic interactions. In the current study, the modulating effect of FB_1 and HFB_1 on the growth and immune indices of the intestinal porcine enterocyte (IPEC-J2) cell line was investigated. The cell survival indices cell viability, apoptosis, and cell proliferation were measured, while inflammatory responses were monitored by immune-detection of interleukin 8 (IL-8). It was found that HFB_1 promotes a greater toxicity on intestinal epithelial cells *in vitro* compared to FB_1. To further investigate the molecular mechanisms and pathways influenced by FB_1 and HFB_1, a comparative proteomic analysis was performed. IPEC-J2 cells were exposed to 7.81 µM and 15.63 µM concentrations of both FB_1 and HFB_1 for 24 hours, respectively. Cells were quantified through proteomic analyses using liquid chromatography- mass spectrometry (LC-MS/ MS). Bioinformatics analyses were conducted and Differentially Abundant Proteins (DAPs) were identified and visualized with volcano plots. The functional annotation of DAPs was carried out using Gene Ontology (GO), comparing data using the *Homo sapiens* and porcine databases. A total of 52 significant DAPs were identified between FB_1 and HFB_1 compared to the control. In the KEGG pathways enrichment analysis, 15.63 µM FB_1 exposure elicited a significant enrichment of proteins within multiple cancer pathways and the AGE/ RAGE signaling pathway. During 7.81 µM HFB_1 exposure, a significant enrichment of proteins was identified within ribosomal pathways, while exposure to 15.63 µM HFB_1 elicited a significant enrichment of proteins within the ECM receptor interaction and proteoglycans in cancer, as well as focal adhesion, and bacterial invasion of epithelial cells. Fibronectin 1 (FN1), an adhesive glycoprotein of the intestine, was the only protein observed amongst all concentrations in cells exposed to FB_1 and HFB_1. It was found that FB_1 up-regulates FN1 and HFB_1 down-regulates FN1, which in turn elicited very different cancer promoting pathways using Cytoscape and STRING enrichment analysis. The expression of FN1 is important in cellular

integrity maintenance, response to intestinal epithelial injury, and wound healing. The potency of FB toxicity on intestinal cells are affected by the complexity of pathways connected. These results suggest that HFB_1 promotes a greater toxicity upon the IPEC-J2 cell line when compared to FB_1, due to the abundance of proteins that were affected during exposure and the interconnectedness of pathways that were enriched.

4.1 Introduction

The intestinal epithelium is regulated by interlinked columnar epithelial cells, such as enterocytes, with critical roles in the intestinal absorptive and digestive maintenance alongside barrier and immune functions (Subramanian *et al.*, 2020). Cellular epithelial tissue is restored every 3-5 days with intricate functions in cell proliferation and apoptosis processes (Pan *et al.*, 2018). The intestinal epithelium is constantly reacting to external harmful exposures, such as bacterial, fungal, or viral microorganisms (MacDonald and Monteleone, 2005). Pathological invasion leads to various forms of infection, which triggers stress response pathways and promotes delamination of cells and increased apoptosis (Apidianakis *et al.*, 2009). Subsequently, a dysregulation of immune and inflammatory signaling is followed, resulting in impaired epithelial renewal and barrier function (Zhou *et al.*, 2017). Therefore, maintenance of intestinal homeostasis is crucial for optimal intestinal functions.

Fumonisin B_1 (FB_1) is a low molecular weight secondary metabolite that causes mycotoxicosis in various mammalian species (Bertero *et al.*, 2018). It has been found that pigs are one of the most susceptible species to FB_1 toxicity and exposure leads to nephrotoxicity, hepatotoxicity, immunotoxicity and disruption of the intestinal barrier function (Devriendt *et al.*, 2009, Halloy *et al.*, 2005, Knutsen *et al.*, 2018a, Knutsen *et al.*, 2018b, Loiseau *et al.*, 2007, Terciolo *et al.*, 2019). FB_1 can also be enzymatically converted to form hydrolyzed fumonisin B_1 (HFB_1), a less potent inhibitor of ceramide synthase, and was found to have controversial results in various *in vitro* and *in vivo* models (Humpf *et al.*, 1998, Schelstraete *et al.*, 2020). However, the mechanisms behind these controversial results, have not yet been fully understood (Caloni *et al.*, 2005, Dellafiora *et al.*, 2018, Hartl and Humpf, 2000, Wang *et al.*, 2016b).

The toxic effects elicited by fumonisins (FBs) may be identified microscopically, where any changes in the functionality and integrity of DNA (genomics), RNA (transcriptomics), proteins (proteomics), or small metabolites (metabolomics) may be observed visually on cells (Eshelli *et al.*, 2018, González-López *et al.*, 2021). In this regard, the use of omics studies is used to identify, characterize and quantify biological tissues and molecules through its structure and function, to obtain a further understanding of toxicity mechanisms that might be harmful to animals and humans (Cimbalo *et al.*, 2022). Proteomics is presented by the structure and function of proteins alongside protein modifications, interactions, and quantification, providing an elaboration of information obtained from other omics technologies (Aslam *et al.*, 2016, Piñeiro *et al.*, 2015). The identification of various signaling pathways provided after protein analysis and enrichment,

contributes to the increased understanding of the mechanisms behind pathogenic interactions and cellular responses (Kan *et al.*, 2017). Although various studies focused on FB_1 and HFB_1 research individually, not many have identified the *in vitro* comparative mechanisms behind the variation of interactions at proteomics level. The interactive mechanisms are important, as these toxins do not occur in isolation in biological systems.

In the current chapter, the analysis, enrichment and identification of specific proteins and pathways affected by FB_1 and HFB_1 exposure upon the porcine intestinal cell line, IPEC-J2, will be focused on and discussed.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Dulbecco's Modified Eagles medium-Ham's F-12 (DMEM HAMS F-12), fetal bovine serum (FBS), epidermal growth factor (EGF), and insulin-transferrin-selenite (ITS) were obtained from Gibco-Life Technologies, (Paisley, UK). L-glutamine, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer, trypsin, Hank's buffered salt solution (HBSS), Penicillin-Streptomycin-Amphotericin B (Pen/Strep/Amph), and Dulbecco's Phosphate Buffered Saline (DPBS) were obtained from Lonza (Basel, Switzerland). Tissue culture flasks were obtained from Lasec (Cape Town, SA) and 60 mm petri dishes from Corning Incorporated (Maine, USA).

Fumonisin B_1 and hydrolyzed fumonisin B_1 were supplied by the Applied Microbial and Health Biotechnology Institute (AMHBI), Cape Peninsula University of Technology (CPUT), (Cape Town, SA), and dimethyl sulfoxide (DMSO) obtained from Sigma-Aldrich (Missouri, USA).

Protein extraction materials include liquid nitrogen (LN_2) purchased from Tygerberg Hospital (Cape Town, SA) and methanol (MeOH) from Labchem (Gauteng, South Africa). Microcentrifuge tubes were obtained from Simport scientific (Quebec, Canada). Acetone, tert-Butyl methyl ether (MTBE), ammonium acetate, triethylammonium bicarbonate buffer (TEAB), guanidine hydrochloride (Gu-HCL), octyl β-D-glucopyranoside (OGP), chloroform, and other analytical grade chemicals were all purchased from Merck/ Sigma-Aldrich (Johannesburg, SA).

Reagents used by the Centre of Proteomic and Genomic Research (CPGR) for proteomic analysis include formic acid, triethylammonium bicarbonate (TEAB), sodium dodecyl sulphate (SDS), ammonium acetate, dithiothreitol (DTT), iodoacetamide (IAA), LoBind plates, and bicinchoninic acid assay (BCA assay) obtained from Merck/Sigma-Aldrich (Johannesburg, SA). Acetonitrile (ACN) and LC water were purchased from Burdick & Jackson (Michigan, USA). HILIC beads were obtained from ReSyn Biosciences (Gauteng, SA), trypsin from Promega (Wisconsin, USA), and Lys-C protease from Pierce (Massachusetts, USA).

4.2.2 Cell Culture studies

4.2.2.1 Intestinal porcine enterocytes

Non-transformed, secondary intestinal enterocytes (IPEC-J2) were gifted from Dr Elisabeth Mayer, University of Innsbruck (Biomin, Tulln, Austria). Cells were inoculated and maintained using methods previously mentioned in section 3.2.2.1.

4.2.2.2 Cell culture

Upon reaching 70-80 % confluency, cells were washed with HBSS, trypsinated and seeded (5 mL) in supplemented maintenance media at the density of 1.5×10^6 cells per dish in 60 mm clear petri dishes. Thereafter, cells were incubated for a minimum of 24 hours at 37 °C in 5 % CO_2/ 95 % air, before media was discarded, and treated (4 mL) with the desired concentrations of FB_1 and HFB_1, made up in 0.5 % FBS DMEM HAMS F-12 media with 1 % DMSO (additional supplementation provided in Appendix 1). The control well contained only 0.5 % media with 1 % DMSO and cells were incubated for an additional 24 hours before commencing with experiments. Eight replicates per treatment were extracted, and a total of 40 samples were prepared and sent to the Centre for Proteomic and Genomic Research (CPGR) for proteomics analyses.

4.2.3 Exposure of cells to FB_1 and HFB_1

Purified and extracted FB_1 (> 95 % purity) and HFB_1 (> 98 % purity) were weighed (21.6 mg/ mL and 5 mg/ mL respectively) and dissolved in DMSO forming a stock solution of 1 mg/ mL. A 31.25 µM concentration was made up in 0.5 % FBS DMEM HAMS F-12 media with 1 % DMSO, sterilized using 0.22 µM corning syringe filters, and diluted into various concentrations, selected from the optimized model development (15.63 µM and 7.81 µM FB_1; 15.63 µM and 7.81 µM HFB_1). The final DMSO concentration did not exceed 1% for all treatments. Eight replicates were used for each concentration for a total of 40 samples.

4.2.4 Sample preparation, protein extraction and clean-up

A SIMPLEX protein extraction method by Coman *et al.* (2016) described below, was modified and used to extract proteins prior to proteomic analysis at CPGR. Cells were prepared and treated as previously mentioned in section 4.2.2 and 4.2.3. Cells were harvested after a 24-hour exposure to FB_1 and HFB_1. Media was discarded and cells were washed with HBSS, trypsinated and transferred into 2 mL Eppendorf tubes. The samples were centrifuged for 5 minutes (500 rpm),

and the supernatant discarded. Consequently, the samples were washed with 1 mL DPBS, centrifuged for 5 minutes (500 rpm), the supernatant discarded, and placed on ice.

For the protein extraction, ice cold methanol (225 µL) was added to the sample pellets. It was then vortexed for 20 seconds and incubated in liquid nitrogen (LN_2) for one minute. The samples were thawed on ice for 5 minutes and placed into a sonicator (Branson Ultrasonics Corporation, Connecticut, USA) for 10 minutes at 4 °C. The LN_2, ice and sonication incubation cycle were repeated three additional times for a total of 4 cycles (LN_2 – ice– sonicate) for complete cell lysis. Thereafter, MTBE (750 µL) was added to the samples, followed by a 1-hour incubation at 4 °C under gentle agitation using a rotator (Stuart, Staffordshire, UK). Subsequently, 0.1 % ammonium acetate (188 µL) was added to the samples to induce phase separation and centrifuged (10 000 rpm) for 5 minutes. The upper lipid fraction was discarded and acetone was added to the remaining fraction (4:1, v/ v). The samples were then precipitated overnight at -20 °C. Samples were then centrifuged for 12 minutes (15 000 rpm) at 4 °C. The metabolite fraction (supernatant) was discarded, and the pellets dissolved in 100 mM TEAB containing 4M Gu-HCL and 1 % OGP (200 µL). The samples were then placed in a sonicating water bath for 5 minutes at 4 °C. Cold methanol (800 µL) was added and vortexed for 20 seconds. This was followed by the addition of chloroform (200 µL) and molecular grade water (600 µL). The samples were vortexed for 20 seconds after each chemical was added. Samples were then centrifuged at 12 000 rpm for 10 minutes at 4 °C and the top aqueous layer discarded. An additional 800 µL of methanol was added to the protein layer and vortexed. The samples were then centrifuged for 10 minutes (12 000 rpm) at 4 °C, and the supernatant, discarded. The pellet was left to air dry on ice for 30 minutes, and samples were stored at -20 °C until analyses.

4.2.5 Proteomic analysis

4.2.5.1 Protein solubilization and quantification

The proteins were solubilized by adding 4 % Sodium dodecyl sulphate (SDS), 100 mM TEAB and heated to 95 ˚C for 10 minutes. This was followed by centrifugation at 10 000 rpm for 10 minutes and the supernatant transferred to a new tube. Thereafter protein quantification was conducted using the bicinchoninic acid assay (BCA assay) (Merck/ Sigma-Aldrich (Johannesburg, SA) according to the manufacturer's instructions.

4.2.5.2 On-bead HILIC digest

The HILIC magnetic bead workflow was prepared by aliquoting HILIC beads into a new tube and removing the shipping solution. The beads were then washed with 250 µL wash buffer containing 15 % ACN and 100 mM ammonium acetate (pH 4.5) for one minute. The beads were washed again for a total of two washes and resuspended in a loading buffer consisting of 30 % ACN and 200 mM ammonium acetate (pH 4.5), to a concentration of 2.5 mg/ mL. A total of 20 µg from each sample was transferred to a protein LoBind plate. The protein was then reduced and alkylated by adding 20 mM DTT and 30 mM IAA, which was followed by a 10-minute incubation at 95 °C. Subsequently, HILIC magnetic beads were added at an equal volume to that of the sample at a ratio of 5:1 total protein. The plate was then incubated on a plate shaker (900 rpm) for 30 minutes at room temperature to bind the proteins to the beads. Thereafter, the beads were washed with 500 µL 95 % ACN for one minute, four times. The protein was digested via the addition of trypsin made up in 50 mM TEAB at a ratio of 1:20 total protein and LysC protease, added at a ratio of 1:250 total protein. The plate was then incubated on a plate shaker at 45 °C for two hours. Subsequently, the supernatant containing peptides were removed and dried, and samples were then resuspended in liquid chromatography loading buffer containing 0.1 % FA and 2.5 % ACN.

4.2.5.3 Liquid chromatography- mass spectrometry

Liquid Chromatography-mass spectrometry (LC-MS) analysis was conducted with a Q-Exacive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Massachusetts, USA) coupled with a Dionex Ultimate 3000 nano-Ultra-high Performance Liquid Chromatography (UPLC) system. The data was acquired using Xcalibur (version 4.1.31.9), Chromeleon (version 6.8-SR13), Orbitrap MS (version 2.9 and build 2926) and Thermo Foundations (version 3.1- SP4). The peptides were dissolved in 0.1 % FA and 2 % ACN. Thereafter, approximately 400 ng of peptide (injected per sample) was loaded on a C18 trap column (PepMap 100, 300 µM × 5 mm × 5 µM). The samples were then trapped into the column and washed for 3 minutes before the valve was switched and peptides eluted into the analytical column. Chromatographic separation was performed with a Waters nanoEase (Zenfit) m/ z Peptide Charged Surface Hybrid (CSH) C18 column (75 µM × 25 cm × 1.7 µM). The solvent system used was solvent A consisting of liquid chromatography water and 0.1 % FA, and solvent B consisting of ACN and 0.1 % FA.

The multi-step gradient for peptide separation was generated at 300 nL/ min as follows: time change 5 minutes, gradient change: 2 – 5 % Solvent B, time change 40 minutes, gradient change 5 – 18 % Solvent B, time change 10 minutes, gradient change 18 – 30 % Solvent B, time change

2 minutes, gradient change 30 – 80 % Solvent B. The gradient was then held at 80 % Solvent B for 10 minutes before returning it to 2 % Solvent B for 15 minutes. All data acquisition was obtained using Proxeon (Theromo Fisher Scientific, Massachusetts, USA) stainless steel emitters.

The mass spectrometer was operated in positive ion mode with a capillary temperature of 320 °C and the applied electrospray voltage was 1.95 kV.

4.2.6 Data analysis for protein identification

The raw data generated was searched against a porcine reference proteome (Pig_Refprot_49792_UP000008227_061221.fasta), downloaded from UniprotKB on 06/12/2021. The raw files were processed using Progenesis QI for Proteomics (Non-linear Dynamics, Newcastle upon Tyne, UK) software and the valid proteins (filtered results to remove reverse hits, common contaminants and singly charged ions) containing at least two unique peptides were reported. A list of regulated proteins (which have the same criteria as valid proteins and in addition are also filtered such that their q-value < 0.05).

Relative quantification was conducted using Progenesis QI for Proteomics (version 2.0.5556.29015) (Nonlinear Dynamics, Newcastle upon Tyne, UK). The data processing included peak picking, run alignment and normalization (singly charged spectra were removed from the processing pipeline). Protein quantitation was run using the “relative quantitation using non-conflicting peptides” method. Database interrogation was performed with Byonic Software (version 3.8.13) (Protein Metrics, Cupertino, USA) using the porcine reference proteome mentioned above.

4.2.7 Bioinformatics analysis

The raw proteomic data obtained from CPGR were further analyzed by Dr Nashia Deepnarain for further bioinformatics analysis to obtain valid proteins and pathways affected by FB_1 and HFB_1 exposure compared to the controls.

The statistical analyses were performed to assess the significance of the observed fold changes between the treatment and control groups. The Benjamini-Hochberg (False Discovery Rate (FDR)) method was used for multiple testing (Benjamini and Hochberg, 1995). Benjamini and Hochberg introduced a method for controlling FDR, which is herein termed BH adjustment. The FDR-based control is less stringent with the increased gain in power and has been widely used

in cases where a large number of hypotheses are simultaneously tested. For hypothesis testing, a T-test was used to produce the gene list after ranked *p*-value and then adjusted *p*-value.

The statistical analyses were performed in R (version 4.2.2) to assess the significance of the observed fold changes between HFB_1 and FB_1 exposures, and the control groups. Differential gene expression was considered significant for the adjusted *p*-value, which was then corrected for multiple testing (FDR) < 0.1 and fold change (FC- mean expression in the experimental group divided by the mean expression in the control group) **≥ 1.3 and ≤ 0.7**; this is represented as the Differentially Abundant Proteins (DAPs*).* To visualize the DAPs, volcano plots with FC values on the *x*-axis and −Log10 (using the adjusted p-values) on the *y*-axis were plotted using the ggplot2 and ggrepel packages in R.

4.2.7.1 Functional annotation and pathway analyses

Functional annotations for DAPs were carried out by gene ontology (GO) to assess the biological processes in R, while using the following packages: DESeq2 and AnnotationDbi packages which ran in the background, clusterProfiler tool package that performed the GO analysis, **org.Hs.eg.db** package was used as the ***Homo sapiens*** **database** of proteins which derive from human cells or tissues, and the **org.Ss.eg.db** package was used as the **Porcine database**, specific to a certain type of genomic annotation or identifier mapping for *Sus scrofa*. These databases are often used to map and annotate biological data, which typically contain information about the protein function.

4.2.7.2 GO and KEGG pathway analysis

Gene Ontology (GO) analysis was performed on the Cytoscape software for further evaluation of the functional enrichment attributes, to annotate the identified proteins in terms of biological process (BP), molecular function (MF) and cellular component (CP) of the identified DAPs. Similarly, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed to predict pathways based on the KEGG database. The GO and KEGG pathways with the adjusted *p*-value (FDR) < 0.05, denoted pathways with significant increases for FB_1 (15.63 µM), HFB_1 (7.81 µM), and HFB_1 (15.63 µM) (Unfiltered pathways provided in supplementary tables 1,2 and 3 respectively).

4.2.7.3 Analysis of the protein-protein interactions networks

The STRING (Search Tool for the Retrieval of Interacting Genes/ Proteins) database (https://string-db.org) was utilized to retrieve the functional protein–protein interaction (PPI) networks of the identified significant DAPs from each data set separated into specific exposure

concentrations. The data originated from various sources which includes automated text mining of scientific literature, computational interaction predictions from co-expression, and databases of interaction experiments and known complexes/ pathways from curated sources. STRING utilizes genomes from authoritative sources only, which includes Ensembl, UniProtKB, Reference Proteomes and the ‘representative genomes’ set in the proGenomes database. The ‘database’ channel imports well-established knowledge about protein complexes, pathways and other functional connections from dedicated knowledge resources including KEGG and Gene Ontology (GO) Complexes (Szklarczyk *et al.*, 2023).

STRING enrichment uses Fisher’s-exact test to calculate the *p*-value and adjusted *p*-value using the BH method for correction of multiple hypotheses testing. The function categories and pathways with p-value < 0.05 were considered significant. Furthermore, additional customization of the PPI network was developed in the Cytoscape software (version 3.10, http://www.cytoscape.org/) to recognize the target proteins by fold change and clustering methods. The top 50 DAPs with significant PPI were selected for further assessment.

4.3 Results and discussion

4.3.1 The effects of FB_1 and HFB_1 on the porcine cell line, IPEC-J2.

In the current study, IPEC-J2 cells were exposed to 7.81 µM and 15.63 µM FB_1 and HFB_1 for 24 hours compared to unexposed control cells. The chosen concentrations were determined following cell optimization experiments conducted as outlined in Chapter Three (section 3.3 and 3.4 respectively). The concentrations selected had no significant adverse effect on cell viability and cell proliferation while cell death and inflammation were slightly but not significantly ($p > 0.05$) increased. Proteins expressed during exposure to FB_1 and HFB_1 were compared, and specific affected pathways were identified to elucidate the mechanisms underlying the intestinal toxicity exerted by FB_1 and HFB_1.

Table 4: The top 52 DAPs between fumonisin B_1 (FB_1) and hydrolyzed fumonisin B_1 (HFB_1) compared to the control. Fold change (FC) values in bold-represent upregulated proteins > 1.3; values greater than 1.3 indicate upregulation, and values less than -1.3 indicate downregulation.

	Protein Symbol	Protein Name	HFB_1 (7.81 µM)		HFB_1 (15.63 µM)		FB_1 (15.63 µM)	
			adjusted *p*-value	FC	adjusted *p*-value	FC	adjusted *p*-value	FC
1	CDK6	Cyclin-dependent kinase 6	0.056	**1.637**				
2	ARFGEF1	ADP ribosylation factor guanine nucleotide	0.047	**1.581**				
3	ACTN2	Actinin alpha 2	0.081	**1.531**				
4	FTL	Ferritin light chain	0.015	**1.500**	0.003	**1.496**		
5	CEP192	Centrosomal protein	0.057	**1.458**				
6	VEZT	Vezatin, adherens junctions transmembrane protein	0.075	**1.424**				
7	MRPL18	Mitochondrial ribosomal protein	0.066	**1.416**				
8	TBC1D23	TBC1 domain	0.077	**1.377**				
9	GCA	Grancalcin	0.059	**1.370**				
10	PGPEP1	Pyroglutamyl-peptidase I	0.046	**1.359**				
11	GOLT1B	Golgi transport 1B	0.083	**1.355**				
12	FTH1	Ferritin heavy chain	0.031	**1.350**	0.004	**1.535**		
13	PDCD10	Programmed cell death protein	0.047	**1.347**				
14	MRPL28	Mitochondrial ribosomal protein	0.049	**1.343**				
15	ABRACL	ABRA C-terminal like	0.072	**1.337**				
16	RPS21	40S ribosomal protein S21	0.057	0.754				
17	CYB5B	Cytochrome b5 heme-binding	0.071	0.752				
18	NFU1	NFU1 iron-sulfur cluster	0.058	0.745				
19	SKP1	S-phase kinase	0.082	0.740				
20	CD44	CD44 molecule	0.058	0.732				
21	WDR48	WD repeat domain	0.045	0.724				
22	TINAGL1	Tubulointerstitial nephritis antigen like 1	0.004	0.716	0.032	0.739		
23	AGRN	Agrin	0.055	0.699				
24	NTN4	Netrin 4	0.048	0.698	0.001	0.549		
25	ATP5F1D	ATP synthase subunit delta	0.045	0.694				
26	RPS28	40S ribosomal protein	0.028	0.690				

27	S100A11	Protein S100-A11	0.050	0.683				
28	SRBD1	S1 RNA binding domain 1	0.032	0.677				
29	SH3BGRL3	SH3 domain-binding glutamic acid	0.048	0.663				
30	FN1	Fibronectin 1	0.003	0.647	0.000	0.585	0.049	**1.371**
31	PPP1R2	Protein phosphatase 1 regulatory inhibitor subunit	0.087	0.642				
32	INTS3	Integrator complex subunit 3	0.094	0.639				
33	SDC4	Syndecan-4	0.005	0.629	0.001	0.623		
34	ST14	Suppressor of tumorigenicity 14	0.006	0.613	0.004	0.586		
35	CLNS1A	Chloride nucleotide-sensitive	0.044	0.607				
36	SPARC	Secreted protein acidic and cysteine rich	0.054	0.605	0.093	0.575		
37	CD276	CD276 molecule	0.073	0.599			0.052	**1.469**
38	FBLN2	Fibulin-2	0.003	0.573	0.000	0.547		
39	CHCHD2	Coiled-coil-helix	0.055	0.469				
40	CCN3	Cellular communication	0.078	0.433				
41	RBP4	Retinol-binding protein					0.056	**1.707**
42	TGFBR2	TGF-beta receptor type-2					0.053	**1.378**
43	EHD2	GTPase superfamily					0.006	**1.335**
44	ATF3	Cyclic AMP-dependent transcription factor ATF-3			0.001	**1.368**		
45	LAMC1	Laminin subunit gamma-1			0.032	0.753		
46	CXADR	CXADR Ig-like cell adhesion molecule			0.013	0.750		
47	QSOX1	Quiescin sulfhydryl oxidase			0.000	0.727		
48	CCN1	Cellular communication			0.006	0.723		
49	MET	Hepatocyte growth factor receptor			0.053	0.672		
50	ITM2B	Integral membrane protein 2B			0.033	0.666		
51	TFRC	Transferrin			0.000	0.628		
52	CDH6	Cadherin 6			0.070	0.447		

4.3.2 Identification of differentially abundant proteins by liquid chromatography- mass spectrometry

Protein is the executor of cell functions and the changes in abundance of key proteins implicate the directly significant effects on corresponding cell functions (McArdle and Menikou, 2020). To investigate the molecular mechanisms and pathways influenced by FB_1 and HFB_1 on the IPEC-J2 cell line, a comparative proteomic analysis was performed. Cells were exposed to 7.81 µM and 15.63 µM FB_1 and HFB_1 concentrations, respectively. The intracellular proteins of cells exposed to FB_1 and HFB_1 as well as the untreated control group, were quantified by LC-MS/ MS methods. The proteins with a fold change ≥ 1.3 and ≤ 0.7 (mean value of all compared groups) and *p*-value (t-test of all comparison groups) < 0.1 by comparing each treated group to the control group were defined as the significantly differentially abundant proteins (DAPs). Differential expression analysis of the protein datasets generated the lists of significant DAPs, fulfilling the selection criteria of fold change ≥ 1.3 and ≤ -1.3, and BH adjusted *p*-value < 0.1. The list of total DAPs along with the FC values, and BH adjusted *p*-values are provided in Table 4. These DAPs for all datasets are graphically represented using the volcano plots in Figures 19 (A-D).

A total of 52 DAPs were generated from FB_1 and HFB_1 exposed cells compared to the control (unexposed cells) (Table 4). Cells that were exposed to 15.63 µM FB_1 resulted in 5 up-regulated DAPs and none that were considered significant following 7.81 µM FB_1 exposure (Table 4: Figure 19C and D). A total of 40 DAPs were identified after 7.81 µM HFB_1 exposure, of which 15 proteins were up-regulated, and 25 proteins were down-regulated, meanwhile cells that were exposed to 15.63 µM HFB_1 elicited a total of 3 up-regulated DAPs and 15 down-regulated DAPs (Table 4: Figure 19A and B). One shared DAP was noted across all three concentrations, while one additional distinct DAP was identified between concentrations of FB_1 (15.63 µM) and HFB_1 (7.81 µM). Furthermore, a total of 9 DAPs identified, overlapped between 7.81 µM and 15.63 µM HFB_1 concentrations. These findings indicate that both FB_1 and HFB_1 exert shared and distinct impacts on the functionalities of the porcine IPEC-J2 cell line.

However, the physiological effects of HFB_1 surpass those of FB_1, as evidenced by a more pronounced alteration in protein abundance within cells exposed to HFB_1 compared to those exposed to FB_1. The analysis revealed a subset of proteins that were commonly expressed across both concentrations of FB_1 and HFB_1-exposed cells, warranting detailed discussion in this chapter. Additionally, specific proteins expressed uniquely in cells exposed to individual concentrations will also be briefly addressed.

Figure 19: Effect of HFB_1 (A, B) and FB_1 (C, D) on DAPs, both at the different concentrations 7.81 µM (represented as HFB1_7 and FB1_7) and 15.63 µM (represented as HFB1_15 and FB1_15), in the intestinal porcine cell line IPEC-J2, respectively. The colours indicate threshold of significant proteins which contain BH adjusted p-values ≤ 0.1, and fold-change ≥ 1 or ≤ 1. Significant down-regulated proteins are shown in blue, up-regulated are shown in red, whereas non-significant proteins are shown in grey.

4.3.3 Functional enrichment analysis of DAPs in FB_1 and HFB_1 related to Gene Ontology

The functional enrichment analysis of the significant DAPs was performed on DESeq2 and clusterprofiler packages in R, to further investigate the stringent common and differential biological processes (BP), cellular components (CC), and molecular functions (MF) underlying FB_1 and HFB_1-induced cell damage, by assessment of the Gene ontology (GO) biological terms (Figures 20 and 21).

There were no significant down-regulated DAPs expressed during 15.63 μM FB_1 exposure, as all identified DAPS expressed were up-regulated.

Following 7.81 μM HFB_1 exposure, DAPs that were down-regulated using the *Homo sapiens* database, were mainly enriched in the overview of exocytosis and ribosomal processes (Figure 20A). Additionally, following 15.63 μM HFB_1 exposure, down-regulated DAPs that were enriched using the *Homo sapiens* database included autolysosome, lysosome and ferric acid binding processes (Figure 20B). Furthermore, using the porcine database, down-regulated DAPs that were enriched after 7.81 μM HFB_1 exposure involved Golgi apparatus and ribosomal processes (Figure 20C); whereby 15.63 μM HFB_1 exposure enriched the ribonucleoside monophosphate biosynthetic and metabolic processes, as well as the negative regulation of extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2) cascade (Figure 20D).

The up-regulated DAPs identified within IPEC-J2 cells after 15.63 μM FB_1 exposure were mainly enriched within microtubule-based movement and lysosomal processes when using the *Homo sapiens* database (Figure 21A). Following 7.81 μM HFB_1 exposure, collagen-containing extracellular matrix, basement membrane and various lumen processes were enriched (Figure 21B). After 15.63 μM HFB_1 exposure, ameboidal-type cell migration, cell substrate adhesion, and cell junction assembly processes were enriched (Figure 21C). When utilizing the porcine database for analysis, the up-regulated DAPs following 7.81 μM and 15.63 μM HFB_1 exposure associated with processes involved in protein-containing complex binding and extracellular matrix (ECM) binding with the morphogenesis of a branching epithelium processes respectively (Figure 21D & E).

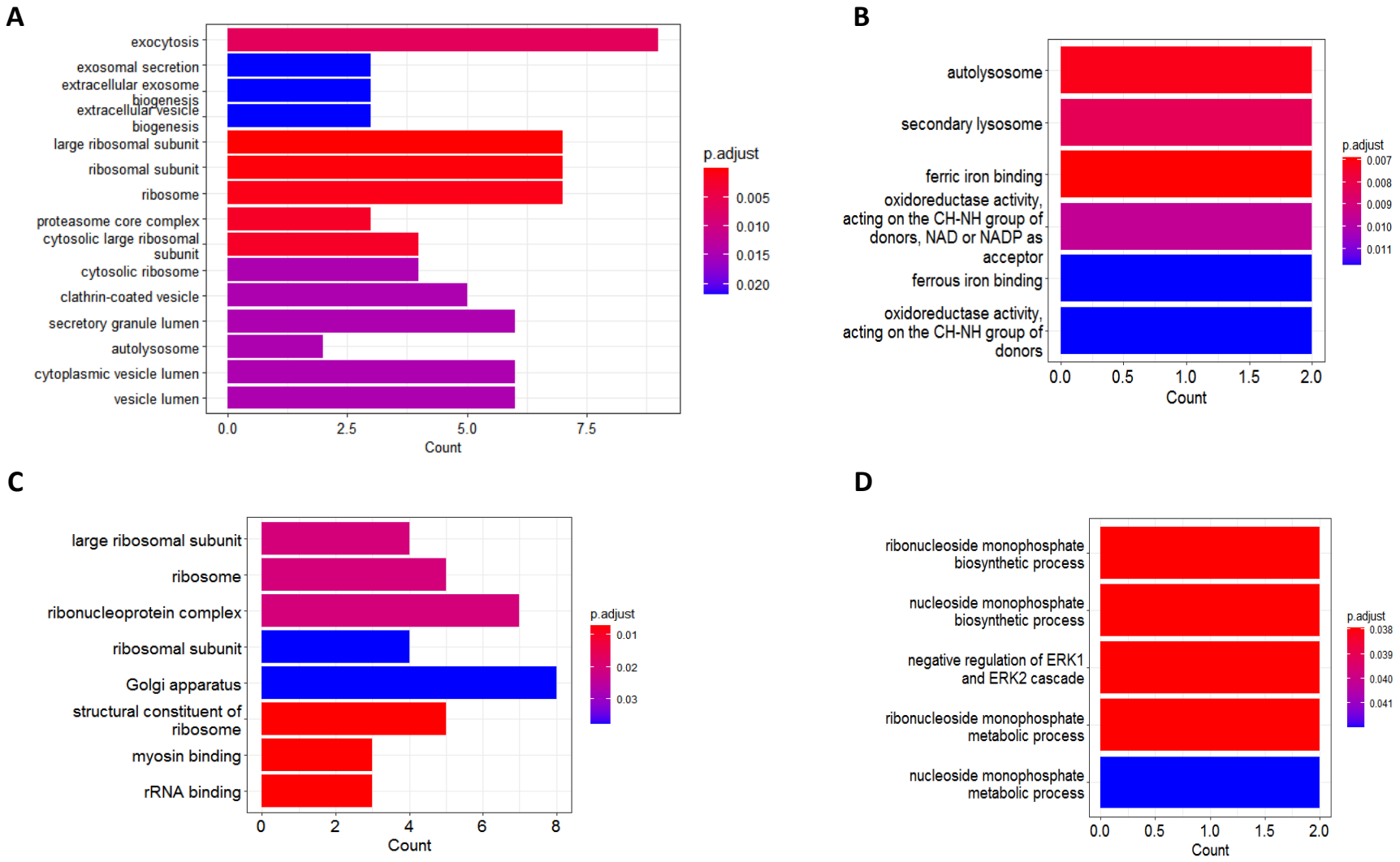
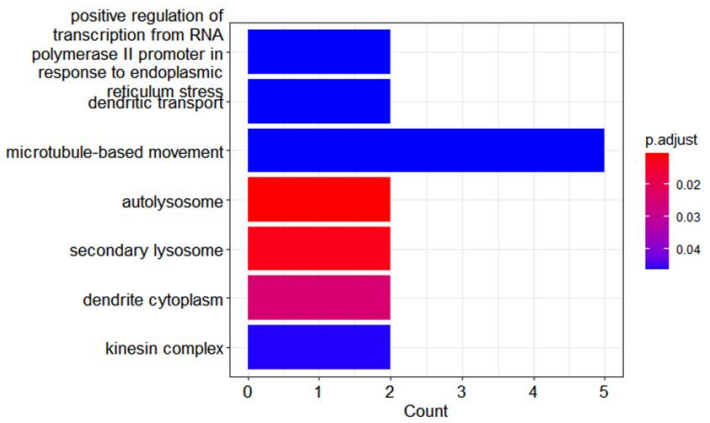
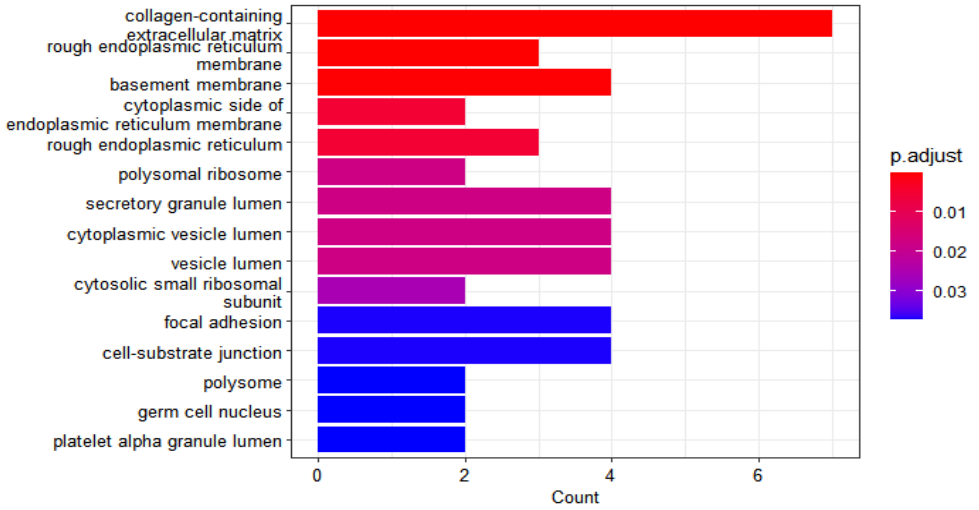


Figure 20: Gene Ontology overview (Biological Process, Cellular Components, and Molecular Function) of the significant **down-regulated DAPs**: A) HFB_1 (7.81 µM) and B) HFB_1 (15.63 µM) using the org.Hs.eg.db- Homosapiens database; C) HFB_1 (7.81 µM) and D) HFB_1 (15.63 µM) using the org.Ss.eg.db -Porcine database. All the GO terms are ranked by adjusted p-value. (Plots generated in cluster profiler in R).

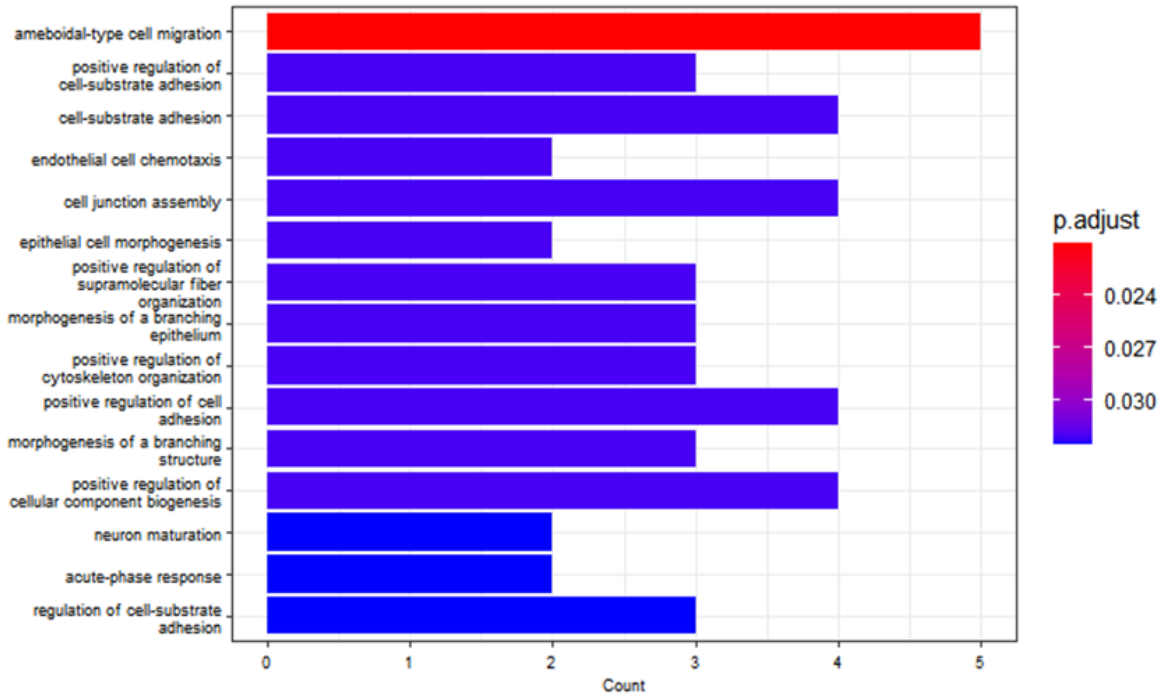
A


positive regulation of transcription from RNA polymerase II promoter in response to endoplasmic reticulum stress
dendritic transport
microtubule-based movement
autolysosome
secondary lysosome
dendrite cytoplasm
kinesin complex
0
1
2
3
4
5
Count
p.adjust
0.02
0.03
0.04

B


collagen-containing extracellular matrix
rough endoplasmic reticulum membrane
basement membrane
cytoplasmic side of endoplasmic reticulum membrane
rough endoplasmic reticulum
polysomal ribosome
secretory granule lumen
cytoplasmic vesicle lumen
vesicle lumen
cytosolic small ribosomal subunit
focal adhesion
cell-substrate junction
polysome
germ cell nucleus
platelet alpha granule lumen
0
2
4
6
Count
p.adjust
0.01
0.02
0.03

C


ameboidal-type cell migration
positive regulation of cell-substrate adhesion
cell-substrate adhesion
endothelial cell chemotaxis
cell junction assembly
epithelial cell morphogenesis
positive regulation of supramolecular fiber organization
morphogenesis of a branching epithelium
positive regulation of cytoskeleton organization
positive regulation of cell adhesion
morphogenesis of a branching structure
positive regulation of cellular component biogenesis
neuron maturation
acute-phase response
regulation of cell-substrate adhesion
0
1
2
3
4
5
Count
p.adjust
0.024
0.027
0.030

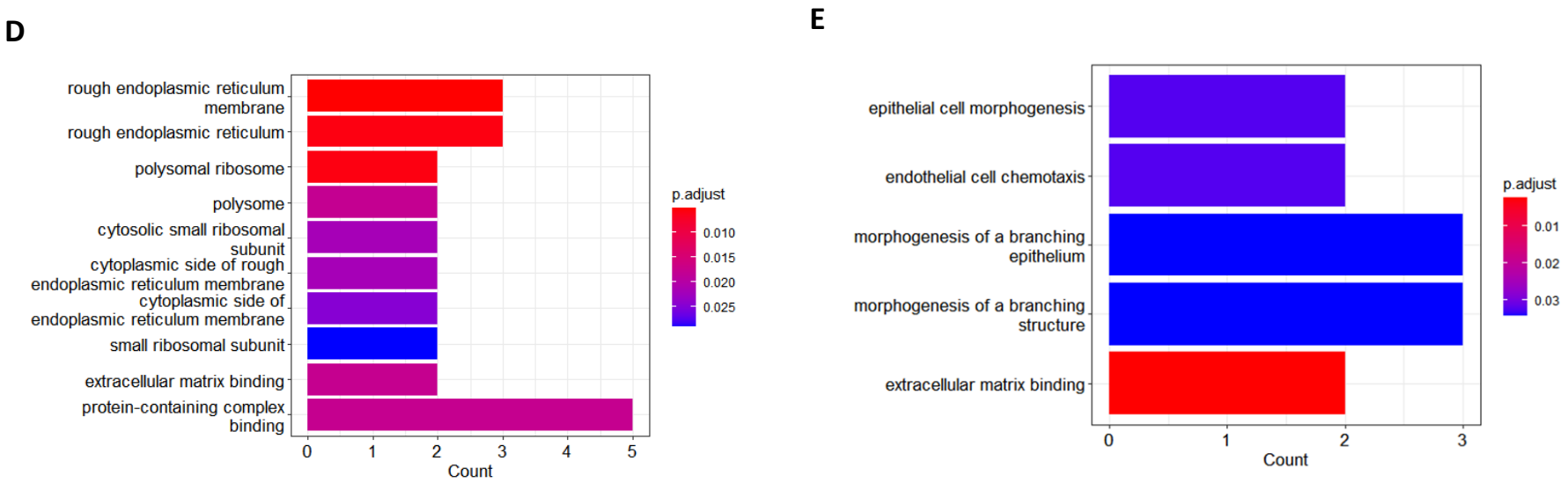


Figure 21: Gene Ontology overview of the significant **up-regulated DAPs**: A) FB_1 (15.63 µM); B) HFB_1 (7.81 µM) and C) HFB_1 (15.63 µM) using the org.Hs.eg.db- Homo sapiens database; D) HFB_1 (7.81 µM) and E) HFB_1 (15.63 µM) using the org.Ss.eg.db - Porcine database. All the GO terms are ranked by adjusted p-value. (Plots generated in cluster profiler in R).

4.3.4 Functional enrichment analysis of DAPs in FB_1 and HFB_1 related to Biological Processes and KEGG Pathways

In the KEGG pathways enrichment analysis (Figure 22), 15.63 µM FB_1 exposure elicited a significant enrichment of proteins within multiple cancer pathways and the AGE/ RAGE signaling pathway. During 7.81 µM HFB_1 exposure, a significant enrichment of proteins was identified within ribosomal pathways, while exposure to 15.63 µM HFB_1 elicited a significant enrichment of proteins within the ECM receptor interaction and proteoglycans in cancer, as well as focal adhesion, and bacterial invasion of epithelial cells.

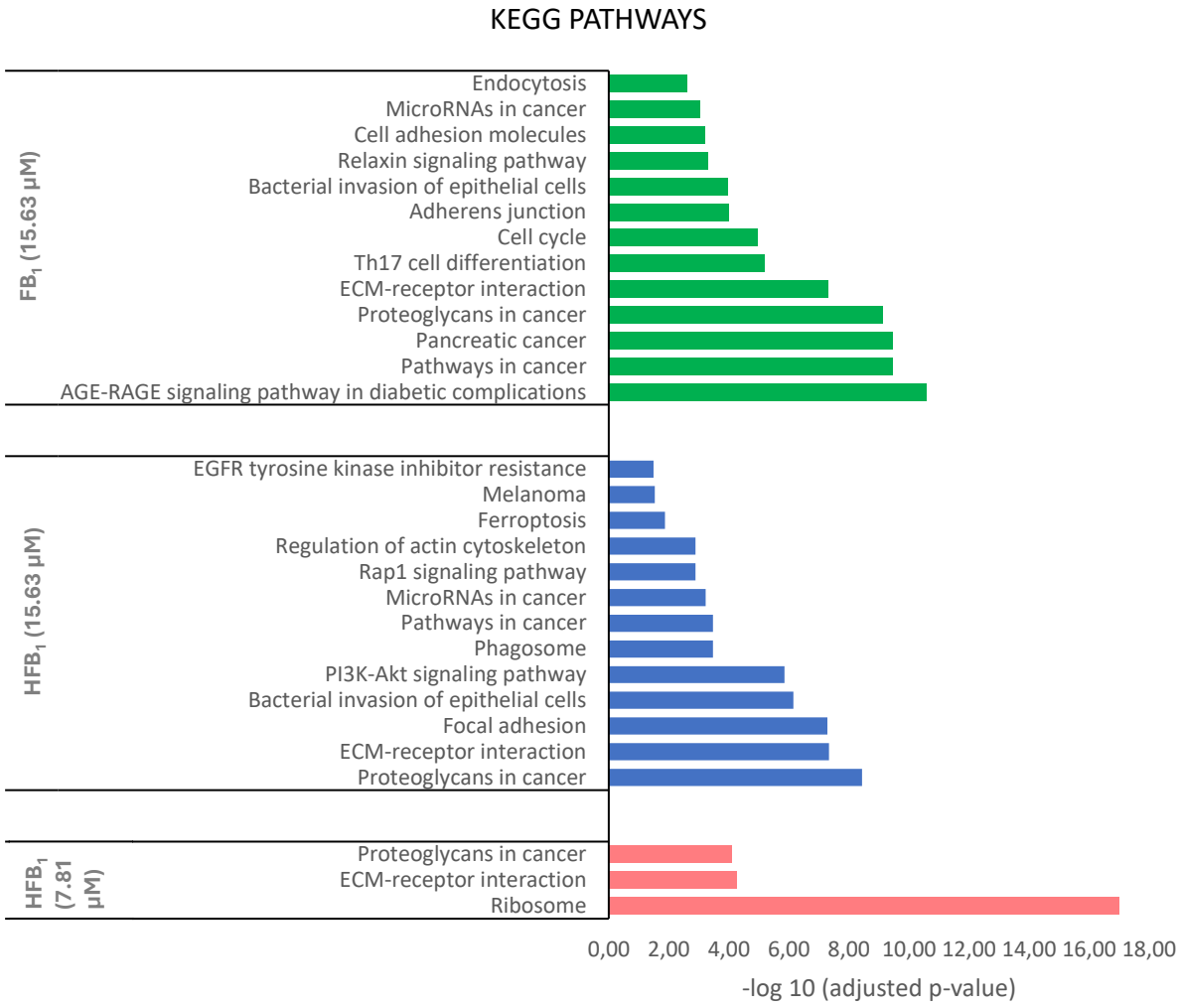


Figure 22: Enrichment analyses of KEGG pathways of the significant enriched proteins associated with FB_1 15.63 µM, HFB_1 15.63 µM and HFB_1 7.81 µM exposed IPEC-J2 cells. All terms are ranked by adjusted p-value.

4.3.5 Differently abundant proteins in IPEC-J2 cells after FB_1 and HFB_1 exposure

Fibronectin 1 (FN1), an adhesive glycoprotein commonly found in multiple epithelial layers of the intestine, was the only protein observed amongst all concentrations in cells exposed to FB_1 and HFB_1. The expression of FN1 is important in cellular integrity maintenance, response to intestinal epithelial injury, and wound healing (Kolachala *et al.*, 2007, Niederlechner *et al.*, 2012, Sun *et al.*, 2020). A study using IEC-6 cells, produced a substantial amount of FN1 under non-stress conditions, but were significantly reduced during intestinal injury, correlating with increased apoptosis (Niederlechner *et al.*, 2012). Similarly in the current study, FN1 was up-regulated during FB_1 exposure and down-regulated during HFB_1 exposure, which suggests that HFB_1 exposure elicits a greater form of intestinal injury compared to FB_1 exposure. Additionally, FN1 has also been associated with the occurrence and development of various tumours by adversely affecting the cell proliferation and migration processes in multiple carcinomas and cancers where FN1 expression was positively related to tumour size (Cai *et al.*, 2018, Lou *et al.*, 2013, Nakagawa *et al.*, 2014, Sponziello *et al.*, 2016, Sun *et al.*, 2020, Waalkes *et al.*, 2010, Wang *et al.*, 2017, Xiao *et al.*, 2018). In the present study, the up-regulation of FN1 during FB_1 exposure could explain why dietary FB_1 initiated rat liver cancer and HFB_1 did not, in the study performed by Gelderblom *et al.* (1993). Furthermore, another protein expressed in 15.63 µM FB_1 and 7.81 µM HFB_1 exposed cells was the CD276 molecule (CD276- also known as B7-H3), which is an immune checkpoint molecule. The CD276 mRNA expression are up-regulated in malignant tumour tissues and higher expression levels are associated with poorer cancer prognosis in patients (Liu *et al.*, 2021). In the current study, CD276 expression was up-regulated at 15.63 µM FB_1 exposure and down-regulated during 7.81 µM HFB_1 exposure, which further suggests that FB_1 affects part of certain cancer promoting pathways but HFB_1 does not.

On the other hand, the up-regulation of ferritin light chain (FTL) and ferritin heavy chain (FTH1) observed with 7.81 µM and 15.63 µM HFB_1 exposure may indicate alternative cancer promoting pathways, as increased FTL and FTH1 expression are associated with increased cancers in humans (Grazi *et al.*, 1995). The down-regulation of netrin 4 (NTN4), after 7.81 µM and 15.63 µM HFB_1 exposure, is also an indication that HFB_1 may be involved in other cancer promoting pathways compared to that of FB_1, as NTN4 it is often down-regulated in the presence of tumours (Esseghir *et al.*, 2007, Latil *et al.*, 2003). Furthermore, the down-regulation of the suppressor of tumourgenicity 14 protein (ST14), a critical tumour suppressor protein found within the gastrointestinal tract, in the presence of HFB_1 also suggests alternative cancer related pathways when compared to FB_1 (Kosa *et al.*, 2012, Netzel-Arnett *et al.*, 2012). It has also been shown

that ST14 is a suppressor of colitis and colitis associated colon carcinogenesis in mice and is expressed mostly within the epithelium (Danielsen *et al.*, 2018). The expression and up-regulation of secreted protein acidic and cysteine rich (SPARC), a matricellular protein, have also been associated with various late stage cancers, adversely affecting cell proliferation, survival, adhesion and migration functions associated with gastric, esophageal and colonic cancers (Brabender *et al.*, 2003, Framson and Sage, 2004, Schiemann *et al.*, 2003, Takemasa *et al.*, 2001, Wang *et al.*, 2004). In the current study, SPARC was down-regulated, suggesting that it may not be associated with the late stage of the above-mentioned cancers. However, it may affect prior stages of other cancers through different pathways.

Additional proteins that were down-regulated with HFB_1 exposure to both concentrations included tubulointerstitial nephritis antigen like 1 protein (TINAGL1), a matricellular protein with a crucial role in regulating angiogenesis by increasing endothelial cell invasion, angiogenic sprouting and sensitivity to transforming growth factor (TGF-β) (Li *et al.*, 2007, Mary *et al.*, 2017, Tajiri *et al.*, 2010). HFB_1 also down-regulated fibulin-2 (FBLN2), a protein that interacts with various ECM ligands and growth factors which supports cell survival and proliferation (Zhang *et al.*, 2020). These findings propose that HFB_1 exposure on IPEC-J2 cells influences angiogenic and extracellular matrix pathways as well. Furthermore, Syndecan-4 (SDC4), a heparan sulphate proteoglycan, commonly found on the surface of endothelial cells, have distinct roles in epithelial wound healing (Bernfield *et al.*, 1992, Pap and Bertrand, 2013). It was observed by Wang *et al.* (2016a), that through ECM and cytokine interactions, SDC4 is expressed during wound recovery and is down-regulated during forms of inflammation such as colitis. This finding coincides with the results obtained from this study as HFB_1 induced inflammation through the induction of IL-8 in Chapter 3 (section 3.3) during exposure.

There were additional proteins that were up- and down-regulated during FB_1 and HFB_1 exposure. The most up-regulated proteins identified during 15.63 µM FB_1 exposure alongside FN1 and CD276 were retinol binding proteins (RBP4), TGF- β receptor type 2 (TGFBR2), and GTPase superfamily (EHD2). The RBP4 protein has an important role in transporting retinol (vitamin A) throughout the circulation and adipose tissues (Perduca *et al.*, 2018). An over-expression of RBP4 is commonly associated with the presence of type 2 diabetes mellitus in humans, and decreased expression impairs immune function through a retinol deficiency (Cho *et al.*, 2006, Fahim *et al.*, 2022, Huang *et al.*, 2018). The significant up-regulation of RBP4 within this study correlates with significant adverse effects within the liver, pancreas and blood vessels as these are also the major organs affected during FB_1 exposure. Up-regulation of TGFBR2, a

transmembrane serine/ threonine kinase that is triggered by TGF-β activation, showed significant associations to cell proliferation, and induction of apoptosis and cellular differentiation pathways (Fynan and Reiss, 1993, Kim, 2001, Markowitz and Roberts, 1996). Similarly in the current study, the same trend was observed during 15.63 µM FB_1 exposure. Furthermore, the up-regulation of EHD2, an ATP-binding and membrane associated protein important in membrane organization and lipid homeostasis regulation, was observed in various hepatocellular carcinoma (HCC) tissues (Shah *et al.*, 2014, Simone *et al.*, 2014, Zhang *et al.*, 2022). Dong *et al.* (2023) also observed an over-expression of EHD2 within the mRNA and protein levels of cirrhotic liver samples, which could be the reason for the production of tumours after FB_1 exposure, within the liver of various animals (Gelderblom *et al.*, 2001).

The three most significantly up-regulated proteins identified during 7.81 µM HFB_1 exposure were cyclin-dependent kinase 6 (CDK6), ADP ribosylation factor guanine nucleoside (ARFGEF1), and actinin alpha 2 (ACTN2). CDK6 is a member of the serine/ threonine protein kinases and are important in cell cycle regulation (progression and transition from G0-S1 phases) (Scheicher *et al.*, 2015). An over-expression of CDK6 was found during enhanced DNA-damage and cell death *in vitro* and *in vivo* within the small intestine, which suggests that HFB_1 exposure to IPEC-J2 cells may have elicited DNA-damage and increased cell death (Chu *et al.*, 2020). The ARFGEF1 protein (previously known as BIG1 protein), have key roles in Golgi integrity, mature integrin β1 glycosylation and neutronium development (Shen *et al.*, 2007, Xu *et al.*, 2022, Zhou *et al.*, 2013). It has been associated with various neurological disorders, however the functionality of the protein is poorly defined (Xu *et al.*, 2022). Up-regulation in the present study suggests an association with some form of neurological damage caused by increased exposure as well. Additionally, ACTN2 is commonly found in the plasma membranes of HeLa cells and mouse myeloma cells (Burridge and McCullough, 1980). It is also highly expressed in cardiac and skeletal muscle, where down-regulation may impact the promotion of cardiac and skeletal muscle defects (Beggs *et al.*, 1992, Lindholm *et al.*, 2021, Murphy and Young, 2015). A direct association between ACTN2 and integrins have been identified, due to the high affinity of integrin binding sites combining with that of ACTN2 (Pan *et al.*, 2018, Qi *et al.*, 2015). It was observed that ACTN2 has a key role in the transmitting force of integrins and the cytoskeleton in mature adhesion (Roca-Cusachs *et al.*, 2013). Therefore, any alteration of ACTN2 expression levels, will most likely affect integrin functions as well (Van der Flier and Sonnenberg, 2001), thereby affecting processes such as cell proliferation and migration, apoptosis, tissue repair and inflammation (Mezu-Ndubuisi and Maheshwari, 2021). It is suggested that the up-regulation of ACTN2 during 7.81 µM HFB_1

exposure in the current study may be a pre-cursor to integrin activation at increased concentrations of HFB_1. Therefore, at higher concentrations of HFB_1, a significant down-regulation of ACTN2 followed by an up-regulation of integrin proteins would be observed, due to the high affinity binding sites. This would then promote an increase of integrin pathways being affected by higher concentrations of HFB_1 exposure.

The most significant up-regulated proteins identified during 15.63 µM HFB_1 exposure in addition to previously mentioned FTL and FTH1, is a cyclic AMP-dependent transcription factor ATF-3 (ATF3). This protein mediates key cellular stress signals, and is over-expressed upon identification of DNA damage, ER-stress, oxidative stress, infection and carcinogen exposure (Hai *et al.*, 1999, Hai *et al.*, 2010, Zhou *et al.*, 2017). In the current study, significant up-regulation of ATF3 during 15.63 µM HFB_1 exposure suggests an interaction with the above-mentioned stress signals, which explains the rapid increase in cell death and inflammation observed in Chapter 3 (section 3.3).

However, significant down-regulated proteins identified during 7.81 µM HFB_1 exposure in addition to FBLN2, were cellular communication (CCN3), and coiled-coiled helix coiled-coiled helix domain-containing 2 (CHCHD2) proteins. CCN3 (also known as Nov) proteins are matricellular proteins secreted within the ECM (Jun and Lau, 2011, Kular *et al.*, 2011). CCN2 have key roles in angiogenesis, and stem cell maintenance, alongside inflammation and wound healing involvement (Akiyama *et al.*, 2017, Gupta *et al.*, 2007, Lin *et al.*, 2005, Lin *et al.*, 2003, Lin *et al.*, 2010). Additionally, CHCHD2, an anti-apoptotic protein located within the mitochondria and regulates the mitochondrial outer membrane permeabilization (MOMP) during apoptosis. Liu *et al.* (2015) found that apoptotic stimuli elicited a down-regulation of CHCHD2 which interacted with and subsequently suppressed pro-apoptotic proteins (Bcl-xL and Bax), allowing MOMP and apoptosis to proceed. This finding could explain why 7.81 µM HFB_1 exposure initiated a dose-dependent increase in apoptosis observed in Chapter 3.

The most significant down-regulated proteins observed during 15.63 µM HFB_1 were Cadherin 6 (CDH6), transferrin receptor 1 (TFRC), and integral membrane protein 2B (ITM2B). CDH6 is a transmembrane glycoprotein, important in the morphogenesis of the central nervous system and kidney (Cho *et al.*, 1998, Inoue *et al.*, 2008). Down-regulation of CDH6 suppressed tumour cell progression but was associated with a poor outcome in cholangiocarcinoma patients, which proposes an association between HFB_1 exposure and progression of certain tumours but not others (Goeppert *et al.*, 2016, Zhao *et al.*, 2021). The expression of ITM2B, an anti-tumour

protein in cancer, plays an important role in supporting apoptosis through suppression of proliferating cells (Baron and Pytel, 2017). However, this protein is commonly associated with Alzheimer's disease, neurogenerative disorders, and vascular disease research. The under-expression of ITM2B was observed in esophageal squamous cancer carcinoma (ESCC) compared to controlled groups (Xian *et al.*, 2024). This finding coincides with the possible association between HFB_1 and induction of esophageal cancer. Additionally, TFRC, a transmembrane glycoprotein, affecting mRNA synthesis and degradation, while controlling the concentration of iron absorption into cells (Gammella *et al.*, 2017, Gerstberger *et al.*, 2014, Marchese *et al.*, 2016), mediates endocytosis and enhances absorption of iron ions from ferritin (Forciniti *et al.*, 2020). It is evident that DNA-synthesis, ATP-production and oxygen transportation are dependent on sufficient iron absorption (Abbaspour *et al.*, 2014, Milto *et al.*, 2016). Therefore, the disruption of iron homeostasis is one of the key observations during the progression of malignant cancer cells, which emphasizes the importance of iron absorption within tumour development, survival, proliferation and metastasis (Forciniti *et al.*, 2020, Ludwig *et al.*, 2015). The significant down-regulation observed within this study suggests a decrease in iron absorption within cells, allows for sufficient tumour progression.

4.3.6 Construction of protein–protein interaction network and pathway analysis of DAPs in FB_1 and HFB_1

Cytoscape- STRING enrichment analysis

To identify vital associated proteins in response to FB_1 and HFB_1, protein-protein interaction (PPI) networks among the identified DAPs with exposure to 7.81 µM and 15.63 µM concentrations were constructed by the STRING database. A cut-off score of 0.7 was used to select PPIs with a high confidence. STRING was used to retrieve the PPI pattern and then exported to Cytoscape for further customization. Cytoscape is one of the most widely used open-source network visualization tools for biological network analysis which supports many use cases in molecular and systems biology, genomics and proteomics. It can make effective use of several visual features that can effectively highlight key aspects of the elements of the network. Node and edge attributes are used to represent quantitative proteomics data and interaction features. It can project and integrate global datasets and functional annotations, using resources such as the Gene Ontology to annotate the interacting nodes/ proteins in the network. In this study, protein interaction networks were generated to identify the biological functions and KEGG pathways

associated with DAPs in the porcine cell line, IPEC-J2. Selection criteria were based on the fact that datasets must be from the origin of *Sus scrofa*.

The identification of the common proteins amongst the three exposure concentrations promoted the indication of common and individual pathways affected. The up-regulation of FN1 during 15.63 µM FB_1 exposure, elicited strong connections with integrin subunit alpha 5 (ITGA5), integrin subunit alpha chain V (ITGAV), integrin subunit beta 1 (ITGB1), and integrin subunit beta 3 (ITGB3). Additionally, FB_1 exposure enriched cancer, proteoglycans in cancer and ECM-receptor interaction pathways (Figure 23). Integrins are a family of transmembrane receptors and that can bind to various ligands within transduction pathways and function in various cell-cell and cellular ECM adhesion (Deng *et al.*, 2019, Li *et al.*, 2014). The alpha and beta subunits often work in combination to determine sufficient ligand specificities (Van der Flier and Sonnenberg, 2001). The expression of ITGA5, which is often combined with ITGB1, are proteins important in cellular differentiation, development and migration, and have been associated with lung tumour progression (Mostafavi-Pour *et al.*, 2018, Ren *et al.*, 2009, Rivera *et al.*, 2017). A significant association between integrin expression and tumour progression, especially within epithelial cells have been observed, as the up-regulation of integrins αvβ3, α5β1, and αvβ6 have been identified during the progression of certain tumours (Desgrosellier and Cheresh, 2010). Dopavogui *et al.* (2022) have also found that FB_1 exposure adversely affects the matrix cell organization of the jejunum through integrin and actin pathways. Similarly, in the present study FB_1 exposure appears to promote tumor progression through integrin pathways, promoting angiogenesis, as well as further activating targeted cytokines and enhancing cell migration and invasion through transforming growth factor-beta pathways (Desgrosellier and Cheresh, 2010). Suggestive of this is the induction effect on inflammation and suppression of cell proliferation, which seems to be cohesive with the results obtained from Chapter 3.

Down-regulation of FN1 observed following 7.81 µM HFB_1 exposure elicited strong connections with various syndecan and collagen pathways. Syndecan-1 (SDC1), syndecan-2 (SDC2), syndecan-4 (SDC4), and collagen type 1, alpha 1 chain (COL1A1) proteins were influenced during HFB_1 exposure, resulting in enriched proteoglycans in cancer and ECM-receptor interaction pathways (Figure 24). Heparan sulphate proteoglycans are associated with colorectal cancer tumourigenesis, as expression within normal cells are significantly increased, but are found to be reduced in highly progressive cancers (Han *et al.*, 2004, Park *et al.*, 2002, Vicente *et al.*, 2018). Additionally, SDC4 may impact cellular proliferation processes by activating the extracellular signal-regulated kinase (ERK) pathway which elicits cellular proliferation (Chua *et*

al., 2004, Corti *et al.*, 2013, Onyeisi *et al.*, 2021). Collagen proteins, such as COL1A1, are usually altered and mutated during tumour progression to facilitate it (Xu *et al.*, 2019). It also interacts with P53, a tumour suppressor gene, along with many other cancer-associated suppressor genes to support cancer progression processes (Álvarez-Garcia *et al.*, 2019, Jolly *et al.*, 2016, Xu *et al.*, 2019). In the current study, it is indicated that 7.63 µM HFB_1 exposure elicits cancer growth by promoting cellular proliferation, cellular migration, adhesion and cell death, via syndecan and collagen pathways. Unlike FB_1, that targets integrin pathways, the alternative cancer promoting pathway is indicative of why HFB_1 toxicity seems to elicit differently during *in vitro* studies when compared to *in vivo* studies.

Furthermore, the down-regulation of FN1 during 15.63 µM HFB_1 exposure elicited a combination of connections of the previous two concentrations. Cytoscape identified connections from FN1 with integrins (ITGA5, ITGB1), syndecans (SDC4), fibulin (FBLN2), and laminin subunit gamma 1 (LAMC1) proteins, all involved in potent cancer promoting pathways. This identification enriched the PI3K-Akt signaling, proteoglycans in cancer, wound healing, and additional cancer pathways (Figure 25). In addition to integrins and syndecans explained above, FBLN2 was found to be overexpressed in lung adenocarcinoma cell lines (Baird *et al.*, 2013). It was also indicated that FBLN2 interacts with the ECM through integrin, collagen and laminin pathways which supports cellular proliferation which further provides reasoning why HFB_1 at increased concentrations elicit greater toxicity upon cells (Zhang *et al.*, 2020). Previous studies indicates that LAMC1 is up-regulated by TGF-β and that increased expression is associated with tumour progression (Fang *et al.*, 2021). It can also activate NF-kB pathway through transcriptional activation which has been an associated link between tumour progression and the inflammatory microenvironment (Fang *et al.*, 2021, Taniguchi and Karin, 2018). The combination of all the above-mentioned pathways by 15.63 µM HFB_1 exposure provides an indication as to why toxicity on IPEC-J2 cells increases as the concentration of HFB_1 toxin increases. HFB_1 toxicity affects more than one cancer promoting pathway, which increases its potency when compared to the previous FB_1 and HFB_1 concentrations.

It is evident that each individual mycotoxin concentration within this study provided very different connective pathways. However, the outcome of proteins and enriched connective pathways identified following FB_1 and HFB_1 exposure have explained why results obtained from previous FB_1 and HFB_1 exposed cells are very different *in vitro* compared to *in vivo*. In this chapter, it was found that FB_1 up-regulates FN1 and HFB_1 down-regulates FN1, which in turn elicited very different cancer promoting pathways through the use of Cytoscape and STRING enrichment

analysis. Therefore, the potency of FB toxicity on intestinal cells are affected by the variety of pathways connected. The results obtained from the previous chapter (Chapter 3) coincides with the data obtained in the current chapter, which states that HFB_1 promotes a greater toxicity upon the IPEC-J2 cell line, due to the abundance of proteins that was affected during exposure and the pathways that were enriched when compared to FB_1.

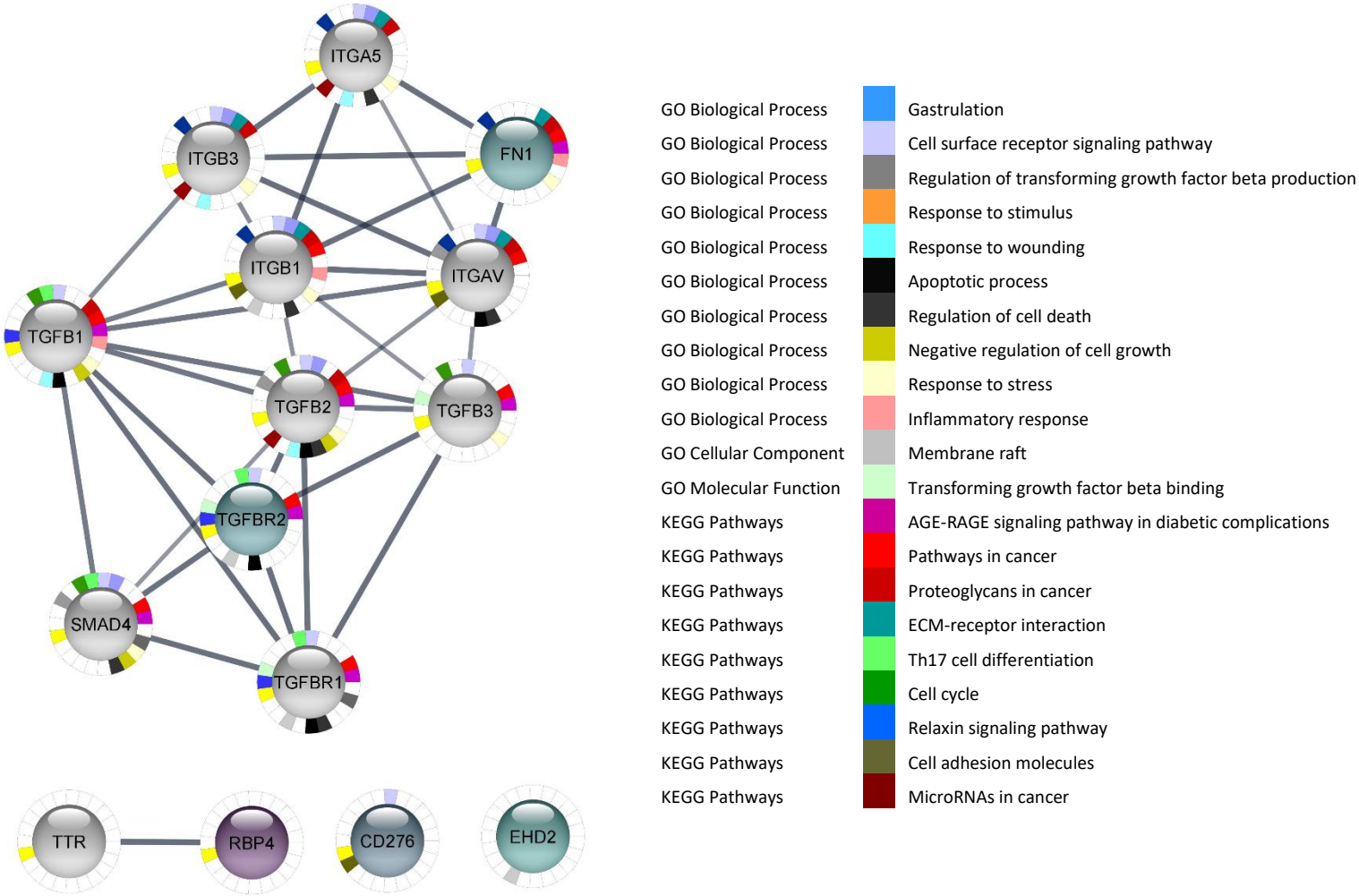


Figure 23: PPI network of FB_1 15.63 µM to determine the signaling pathways of the 5 significant upregulated proteins in darker green to purple. The PPI network consisted of 15 nodes and 32 edges

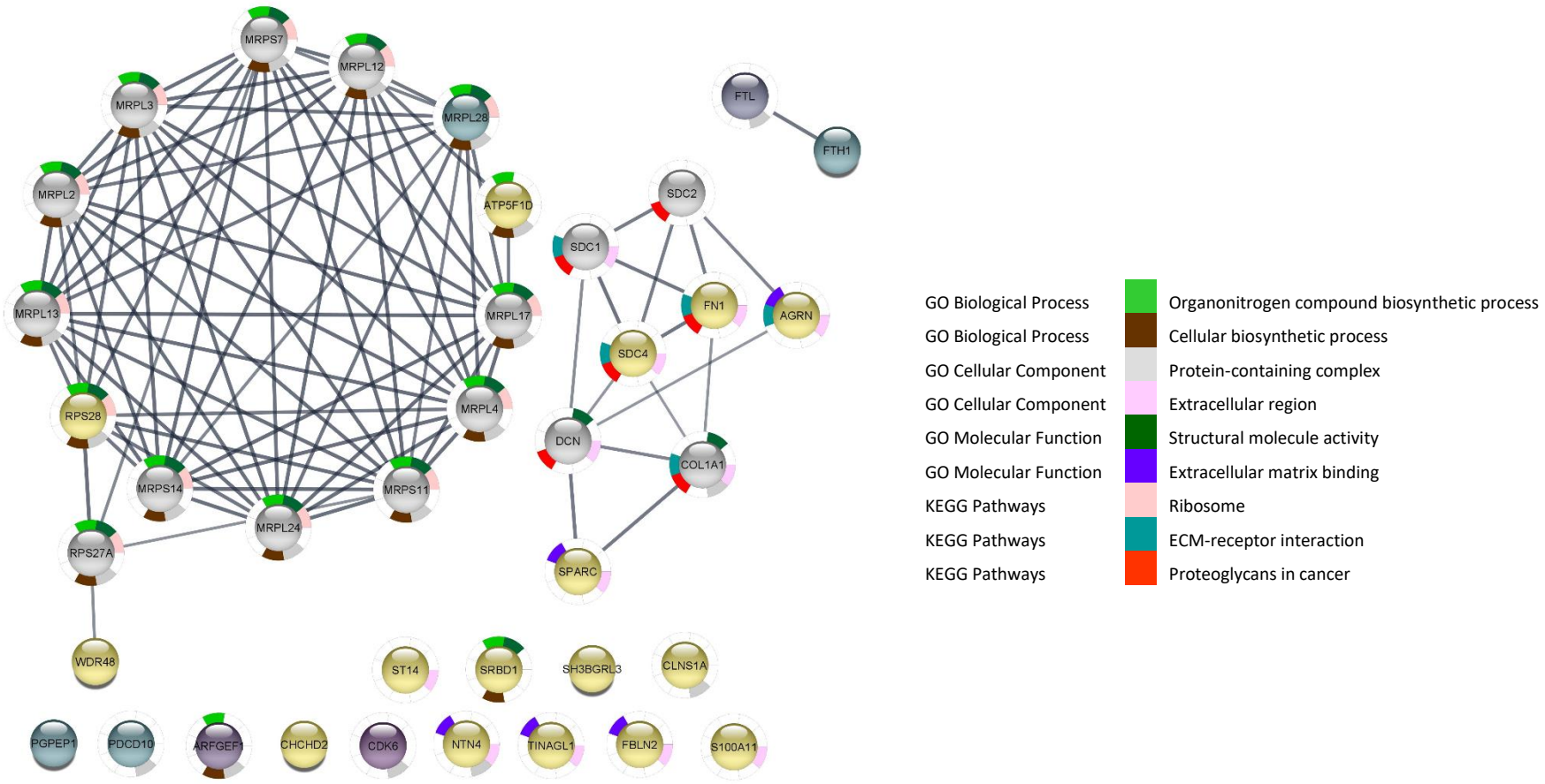


Figure 24: To determine the signaling pathways of the identified 40 DAPs: PPI network of HFB_1 7.81 µM. The PPI network was constructed using string database and customized in Cytoscape and, where each node represents a protein, and edges represent the interaction between proteins. The PPI network consisted of 38 nodes and 85 edges. Down-regulated DAPs in sus scrofa intestinal cell line (IPEC-J2), are shown in yellow whereas the up-regulated are shown in dark green-purple, other predicted proteins shown in grey. Interactions were predicted with a high confidence level of 0.700 and enrichment p-value < 0.05 were included in the analyses.

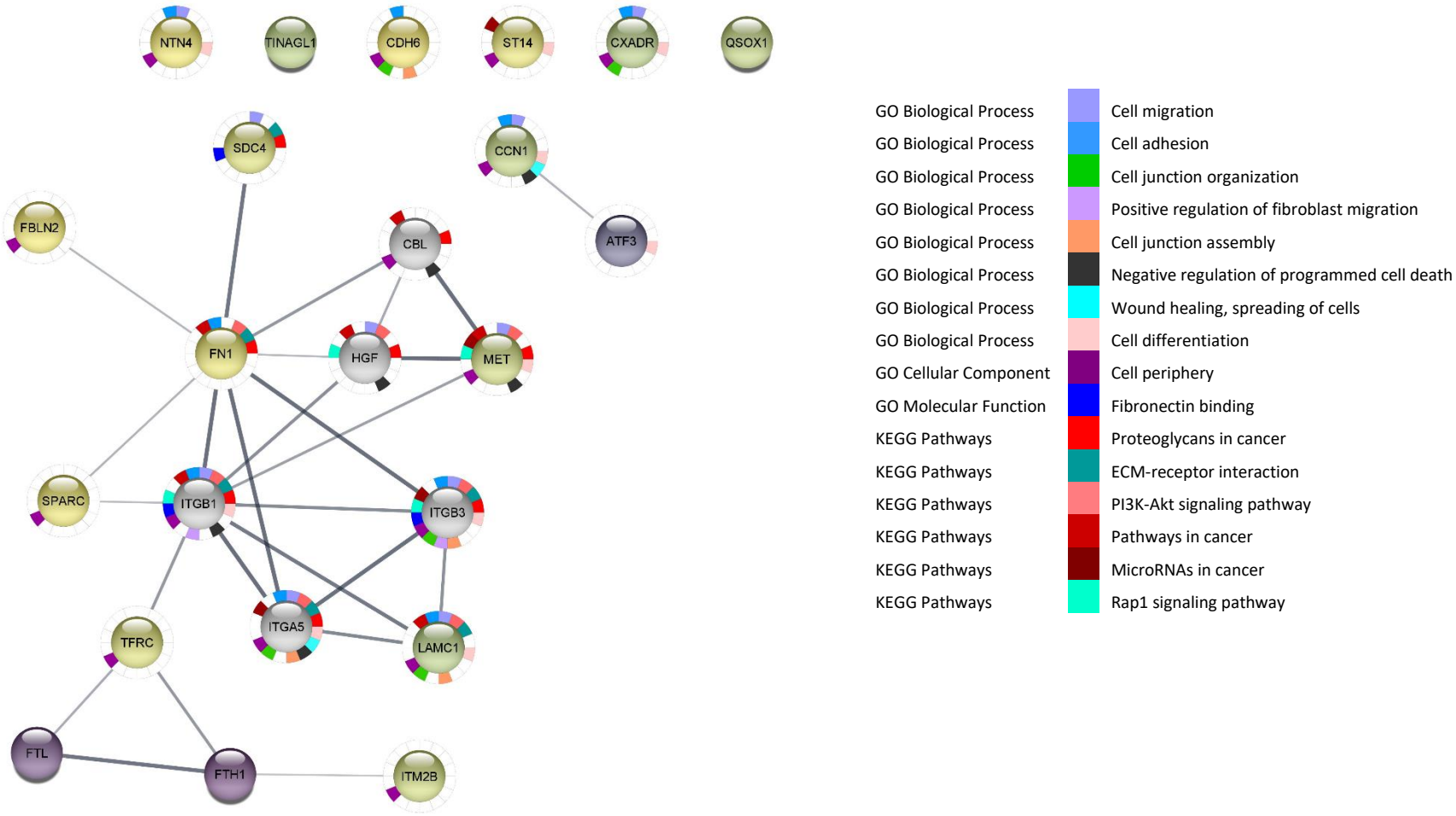


Figure 25: PPI network of HFB_1 15.63 µM, to determine the signaling pathways of the 18 significant proteins identified; significant upregulated proteins in darker purple; whilst down regulated proteins shown in yellow. The PPI network consisted of 23 nodes and 26 edges.

4.4 References

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CHAPTER 5: General Conclusion

5.1 Conclusion

It is evident that fumonisin exposure presents many adverse effects within the intestinal epithelium. In the current study, the optimization of the IPEC-J2 cell line was obtained. This was accompanied by the identification of individual exposure and co-exposed with FB_1, HFB_1 and LPS effects upon the cell line. Following optimization, an analyses and enrichment of affected proteins and pathways during FB_1 and HFB_1 exposure were identified. The individual exposure and co-exposed data obtained from FB_1, HFB_1 and LPS suggests that HFB_1 promotes a greater toxicity upon the intestinal epithelial cells compared to FB_1. HFB_1 is known particularly for its lowered toxicity when compared to FB_1 *in vivo*. However, controversial results obtained from previous studies alongside the novel data obtained from the present study indicate that HFB_1 may be metabolized differently *in vitro* compared to *in vivo*.

Following protein analyses and enrichment, it was further discovered that FB_1 and HFB_1 uses different pathways to exert its toxic mechanisms upon intestinal epithelial cells. The proteins identified within this study briefly explains why FB_1 and HFB_1 affects IPEC-J2 cells so differently. The findings may also explain why HFB_1 elicited controversial results in many *in vivo* studies compared to *in vitro* studies. For instance, the identification of fibronectin 1 being up-regulated by FB_1 and down-regulated by HFB_1 indicates the differential enrichment of various cancer promoting pathways. Both chapters present data which suggests HFB_1 to be more toxic on the porcine intestinal epithelial cells compared to FB_1. This was indicated by the higher abundance of proteins identified and affected during HFB_1 exposure when compared to FB_1, together with the specific pathways enriched. It was also observed that HFB_1 elicited a greater adverse effect on the cell survival indices and inflammatory parameters compared to FB_1. This is also observed in the individual exposure and co-exposure model, where greater cytotoxicity occurred during HFB_1 exposure. These results coupled with that obtained from proteomics further indicates that HFB_1 elicits a greater adverse toxic effect upon the IPEC-J2 cell line compared to FB_1, which may be due to the way it is metabolized in the gut. These results demonstrate the complexity of fumonisin metabolism and effect on the intestinal gut cells as well as the informative use of bioinformatics for analyzing complex data sets.

Appendix 1

Media supplementation:

Materials:

Cell culture materials include Dulbecco's Modified Eagles medium-Ham's F-12 (DMEM HAMS F12), fetal bovine serum (FBS), epidermal growth factor (EGF), and insulin, transferrin and Selenite (ITS) were obtained from Gibco-Life Technologies, (Paisley, UK). L-glutamine, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer, trypsin, Hank's buffered salt solution (HBSS), Penicillin-Streptomycin-Amphotericin B (Pen/Strep/Amph), and Dulbecco's Phosphate Buffered Saline (DPBS) were obtained from Lonza (Basel, Switzerland).

1. **Inoculation (Revival) media:**
   DMEM/ HAM's F-12 media, supplemented with 20 % (v/ v) heat inactivated FBS, 16 mM HEPES, 2.5mM Glutamax, 5 ng/ mL EGF, 1% ITS and 1 % PSF (100 IU/mL penicillin and 100 μg/ mL streptomycin)

2. **Maintenance media:**
   DMEM/ HAM's F-12 media supplemented with 10 % (v/ v) heat inactivated FBS, 16 mM HEPES, 2.5mM Glutamax, 5 ng/ mL EGF, and 1 % ITS

3. **Experimental media:**
   DMEM/ HAM's F-12 media supplemented with 0.5 % (v/ v) heat inactivated FBS, 16 mM HEPES, 2.5mM Glutamax, 5 ng/ mL EGF, 1 % ITS, and 1 % DMSO

Appendix 1: Media supplementation