Deoxynivalenol: toxicological profile and potential for reducing cereal grain contamination using bacterial additives in fermented animal feed.

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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"Where there is no path, leave a trail" Ralph Waldo Emerson

Conferences attended, platform presentations, laboratory visits, successful grant applications, reviewed manuscripts and published articles.

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Seale-Hayne Educational Trust, July 2009- July 2010, Grant application: The screening of lactic acid bacteria derived from wild and domesticated birds for mycotoxin metabolising/binding properties leading to remediation of mycotoxin contamination in fermented liquid animal feeds derived from by-products of bioethanol production. (£4880).

The world mycotoxin forum conference 2010, Noordwijk, Netherlands, 8-10/11/10, poster presentation: Exploring the DON-bioremediation potential of lactic acid bacteria derived from wild birds.

Seale-Hayne Educational Trust, August 2010-August 2011, Grant application: Possible underestimation of toxic Deoxynivalenol in grain-based animal feeds produced in the South West. (£2800).

Reviewed manuscript: *In vivo* genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats, 21/10/11, Regulatory Toxicology and Pharmacology.

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William F. Vevers, Richard Billington, Jane Beal

Abstract

Deoxynivalenol (DON) contamination of grain destined for animal feeds is a major toxicological risk to monogastrics and is suspected of restricting productivity in ruminants. Whereas bacterial additives have been developed that can detoxify DON in the rumen and lower intestine, there are currently no commercial inoculants able to perform this task in crimped grain (CG) silage, a regionally important method of moist grain preservation based on homo- and heterofermentative lactic acid bacteria or chemical additives. Determining whether this ensiling process alongside the action of detoxifying bacteria has the potential to remove DON in CG prior to ingestion, was explored in mini-silo ensiling experiments. CG was heat treated (100 °C, 60 min) or ensiled fresh in triplicate 50 g silos, spiked with 5 mg/kg DON and inoculated with lactic acid bacteria derived from wild birds, natural epiphytic inoculants and commercially sourced silage additives (21 d). DON recovery was only significantly reduced (31.2 ± 14.4% recovery, $p<0.001$, n= 30) by heat treatment, as determined by IAC-RP-HPLC-UV. Bacterial assemblage analysis by 16S rRNA PCR-DGGE-SEQ identified *Weissella cibaria, Pantoea agglomerans, Bacillus subtilis, B. licheniformis* and *Hafnia alvei* as candidate detoxification agents, of which *W. cibaria* and *H. alvei* decreased DON recovery *in vitro* (11.3 and 6.2% recovery respectively, $p<0.05$, n = 18), which translated to inoculated *W. cibaria* yielding a decrease in DON recovery (67.2± 14.4%, 28 d) in naturally contaminated crimped wheat (13.5 ± 1.0 mg/kg, 35-40% moisture, $p<0.05$, n =15). As *W. cibaria* is a lactic acid bacteria already associated with fermented CG by default it has promise as a novel DON detoxification agent in CG silage. DON is however just one of many hepatotoxic co-contaminants. Retrorsine, a DNA-crosslinking pyrrolizidine alkaloid derived from Ragwort (*Senecio* sp.) was investigated for interactive toxicity with DON in an *in vitro* co-exposure experiment. HepG2 cells were exposed to Log_{10} multifactorial binary exposures for 48 h followed by a suite of assays to elucidate mechanisms of interactive cytotoxicity, genotoxicity and modulation of the proteome. Retrorsine was tentatively confirmed to form DNA/protein crosslinks in the comet, micronucleus and crosslinking assays, whilst DON was found to potently induce cytotoxicity and apoptosis. Co-exposure yielded a complex toxicity response, with low doses yielding antagonistic effects and high doses trending towards additive effects, although DON dose was generally the principle component. The difficulties associated with undertaking an interactive toxicity study where both toxins have multiple metabolic and cellular targets are highlighted.

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Abbreviations

15AcDON; 15-acetyl-deoxynivalenol
3AcDON; 3-acetyl-deoxynivalenol
AA; Acetic acid
ABC; Ammonium bicarbonate
ACN; Acetonitrile
AFB1; Aflatoxin B1
ANOVA; Analysis of variance
APH; Aphidicolin
BCA; Bio-control agent
BSA; Bovine Serum Albumin
CBMN; Cytokinesis-blocked micronucleus
CFDA; 5-6,-carboxyfluorescein diacetate
CFS; Cell-free supernatant
CFU; Colony forming units
CG; Crimped grain
CODA; Chloramphenicol/oxytetracyline dextrose agar
Cyto-B: Cytochalasin-B
D3G; Deoxynivalenol-3-β-d-glucoside
Da; Daltons
DAS; Diacetylscirpenol
DGGE; Denaturant gradient gel electrophoresis
DHP; Dehydopyrrolizidine pyrrole derivative
DM; Dry matter
DMSO; Dimethylsulfoxide
DNA; Deoxyribose nucleic acid
DOM-1; De-epoxide-deoxynivalenol
DON; Deoxynivalenol
DPBS; Dulbecco's A phosphate buffered saline
EC50; Effective concentration 50%
EDTA; Ethylene diamine tetraacetic acid
ELISA; Enzyme linked immunosorbent assay
EtOH; Ethanol
FD; Fluorescent detection
FHB; Fusarium Head Blight
FITC; Fluorescein isothiocyanate
F. ; Fusarium
GC; Gas chromatography
GI; Gastro-Intestinal
GLM; General linear model
HPLC; High-performance liquid chromatography

HU; Hydroxyurea
L. ; Lactobacillus
LAB; Lactic acid bacteria
LC50: Lethal concentration 50%
LC-MS; Liquid chromatography mass spectrometry
LDH; Lactate dehydrogenase
Leu. ; Leuconostoc
LF; Liquid feeds
LIMOX; Limonene-1,2-epoxide
LOD; Limit of detection
MEM; Minimal Eagles medium
MeOH; Methanol
MMC; Mitomycin-c
MRS; De Man, Rogosa and Sharpe
MS; Mass spectrometry
MSM; Minimal salts media
MT; Mycotoxins
MTT; 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
NADH/NAD+; Nicotinamide adenine dinucleotide
NDI; Nucleoplasmic division index
PA; pyrrolizidine alkaloids
PAT; Patulin
RCA; Reinforced clostridial agar
RET; Retrorsine
RIPA; Radioimmunoprecipitation assay
RNA; Ribose nucleic acid
ROS; Reactive Oxygen Species
RP-HPLC; Reverse phase high performance liquid chromatography
Sodlac; Sodium lactate (media)
SPE; Solid phase extraction
TFAA; Trifluoroacetic acid
TMR; Total mixed ration
TSB/TSA; Tryptone soya broth/agar
U-HPLC; Ultra high pressure liquid chromatography
UV; Ultraviolet
UVD; Ultraviolet detection
VOD; Veno-occlusive disease
WCS; Whole crop silage
Wks; Weeks
ZEA; Zearalenone

1 Deoxynivalenol: toxicological profile and potential for reducing cereal grain contamination using bacterial additives in fermented animal feed.

1.1 Introduction

Fermented animal feeds including crimped (CG) grain silage make up an important proportion of animal feeds globally with a significant area of agricultural land dedicated to its production. As with all agricultural commodities CG silage can be susceptible to toxin producing pathogens in the field and spoilage organisms in storage (Borling Welin *et al.*, 2015, Driehuis *et al.*, 2008). Beneficial microorganisms, such as lactic acid bacteria (LAB) minimise the latter during the ensiling process, with the production of organic acids which efficiently preserve the nutritive properties of the feed by lowering the pH and inhibiting the growth of undesirable organisms (Dunière *et al.*, 2013). However, this study will examine the additional possibility of such bacteria having the ability to increase feed safety by reducing the amount of pre-harvest toxins such as the ubiquitous mycotoxin (MT); deoxynivalenol (DON), the main subject of this investigation.

1.2 DON: primary role, distribution and molecular structure

Deoxynivalenol (DON) is a sesquiterpenoid mycotoxin (MT) and part of the type B trichothecene group ($C_{15}H_{20}O_6$, 296.32 g/mol). All trichothecenes are characterised by a 12, 13-epoxide group with type B trichothecenes such as DON (Figure 1.1) having a carbonyl group bonded to the C-8 position (Pestka, 2007).

DON is one of thousands of MTs found in a range of harvested staple commodities across the globe (D'Mello and Macdonald, 1997, Hussein and Brasel, 2001, Binder *et al.*, 2007). MTs are toxic secondary metabolites produced by fungi and whilst the majority have yet
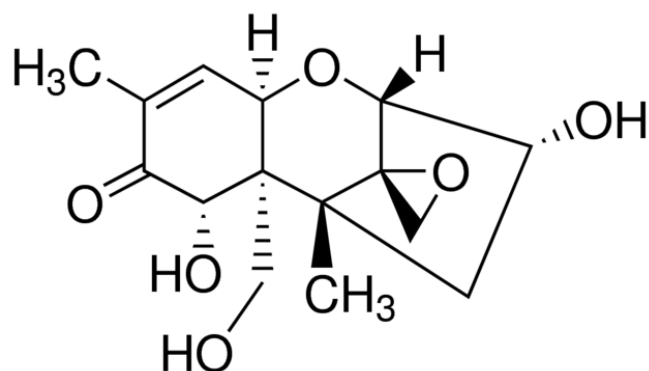
Figure 1.1 Structure of DON (Sigma-Aldrich).

to be conclusively associated to any defined ecological function (Adam *et al.*, 2015), the toxic effects of these compounds to humans and production animals has attracted significant research effort (Hussein and Brasel, 2001). The role of DON in plant-pathogen interactions is a little more understood. Correlational evidence suggests DON serves as a virulence factor in the colonisation of cereal crops (Ilgen *et al.*, 2008), with Jansen and co-workers proposing the inhibition of protein synthesis by DON allows the egress of hyphae between the interstitial space of the grain, and consequently the colonisation of the whole plant head. Although DON is not strictly essential for mould growth, trichothecene gene knockouts did not yield the same degree of egress (Jansen *et al.*, 2005). Also, the mechanism of DON synthesis by the mould has been revealed to be fairly complex involving a large suite of enzymes fulfilling very little other purpose, adding support to the virulence factor hypothesis (Desjardins *et al.*, 1993). However, it must be noted that although the virulence hypothesis is conceivably valid, there is currently a paucity of evidence to support it conclusively (Adam *et al.*, 2015).

The impact of DON contamination on temperate food supply chains due to DON's toxicity profile, ubiquitous distribution and resistant chemical structure has triggered extensive

research into methods of contamination prevention, robust detection and remediation (Jouany, 2007, Speijers, 2003, Binder, 2007, Bhat *et al.*, 2010).

DON is produced by plant pathogenic moulds including; *Fusarium graminearum* and F. *culmorum*, the main causative agents in *Fusarium* Head Blight (FHB) in grain crops and Gibberella ear rot in maize. The wide geographical distribution of both *F. graminearum* and *F. culmorum* across temperate agricultural climates means DON contamination is a constant threat for all grain producers, food/feed manufacturers and consumers (D'Mello *et al.*, 1999). Contamination levels are found to be strongly related to prevailing climatic conditions during crop anthesis (Langseth and Elen, 1997, Wagacha and Muthomi, 2007), although other factors such as land stewardship, use of fungicides and crop resistance all play a part in modulating the final contamination level from year to year (Edwards, 2004, Beyer *et al.*, 2006).

1.3 Detection of DON

Prior to the advent of liquid chromatography which allowed detection of MTs down to µg levels, the presence of deleterious MTs in agricultural commodities was difficult to determine. Indeed, only the historical reoccurring mycotoxicosis episodes in both humans (St Antony's Fire, (Matossian, 1984)) and production animals (Turkey X disease, 100,000 turkeys succumbed to AFB_1), yielded a suggestion of toxigenic mould involvement, with the causative agents only identified and named "mycotoxins" in the early 1960's (Richard, 2007). It must be noted that in many countries, especially in rural sub-Saharan Africa, the provision of adequate MT detection and quantification methods are still scarce leading to human fatalities and severe health risks (Turner *et al.*, 2007). In modern agricultural practices within the EU, the provision of strict MT testing of

commodities to set maximum permitted levels (Table 1.1) has meant that human mycotoxicotic outbreaks are now rare in the developed world with effects on animal productivity the current priority concern, although current guidance for animal feeds is based on commodities at 12% moisture only (see Table 1.2 for guidance maximum levels in the EU (European-Commision, 2006)). A suggestion by Eckard *et al.* (2011) to carry out a Europe-wide analytical survey of whole-crop maize silage, an untraded on-farm commodity outside the realm of MT testing, was supported by the fact that 100% of the maize tested in Switzerland was DON contaminated between 780-2990 μg/kg. Since ensiled maize and cereals form a large proportion of cattle forage in modern agriculture (Driehuis, 2013), it is imperative that robust methods of silage analysis are developed to determine risk and instigate amelioration strategies.

Additionally, guidance values have been recommended for a further five mycotoxins in animal feeds under Commission Recommendation 2006/576/EC: deoxynivalenol, zearalenone, ochratoxin A and fumonisins B1 and B2. These mycotoxins pose a risk to animal health and can affect livestock production for several species, but the risk to public health is considered low; in all cases, food of animal origin only contributes marginally to the total human exposure to these toxins.

Adequate detection and quantification of MTs is reliant on efficient extraction from the commodity matrix, the suitability of the separation protocol and the sensitivity of chosen mode of detection. Reverse Phase High Performance Liquid Chromatography (RP-HPLC) is broadly the industrial standard for separation followed by gas chromatography, although immunoaffinity methods have recently been shown to yield comparable results in the

form of ELISA assays, or used solely for the initial sample clean-up stage for chromatography (Turner *et al.*, 2009).

Table 1. 1. The maximal permitted limits of DON in human food in the European Union (EC No 1881/2006 (Amended by 1126/2007, 105/2010, 165/2010, 594/2012, 1058/2012).

Consumer	Food type	Maximum permitted DON content (mg/kg final product)	Note/exceptions
Human; adult	Unprocessed cereals other than maize, oats and durum wheat	1.25	
	Unprocessed durum wheat, maize and oats	1.75	(exception of maize for wet milling)
	Semi-processed grain products such as barley / wheat / maize flour and flakes	1 (increased from 0.75, 28th Nov. 2013)	A maximum tolerated daily intake of 1 µg/kg body weight is recommended by the EU for consumption of DON in humans.
	Processed foods including bread, biscuits, cereal snacks and breakfast cereals	0.5	
	Pasta (dry)	0.75	
Human; infants	Baby food (dry matter basis)	0.2 (reduced from 0.5, 28th Nov. 2013)	Thought to be at the most at risk from DON

Table 1. 2. The guidance limits of DON in animal feeds in the European Union (data extracted from the Commission Recommendation (European-Commission, 2006a))

Feed type	Feed Ingredients	Maximum permitted DON content at 12% moisture (mg/kg)	Note/exceptions
Part ingredient in animal feed	Barley/wheat	8	Level in complete feed or complementary feed must not exceed 5 mg/kg, or levels stated as follows:
	Maize	12	
Complete feeding material/ complimentary inclusion	Barley/wheat	5	for pigs the maximum level is 0.9 mg/kg , for calves <4months, lambs and kids the maximum level is 2 mg/kg
	Maize	5	

In the current investigation, microbial MT biotransformation will be evaluated in fermented animal feeds and growth media. The extraction and detection protocol needs to be robust against any cross-contaminating agents stemming from the components of bacterial growth, inherent media acidity, the low concentrations of DON used, the additional requirement of detecting DON-metabolites and the sequestering potential of the feed component matrix. Such complex media requires extensive sample clean-up and sensitive detection capacity, as illustrated in Table 1.3. There are relatively few publications describing the extraction of DON from fermented feeds or bacterial media (Driehuis *et al.*, 2008), although this will likely increase along with the expanding interest in this research area (WMF, 2014).

Seeling and co-workers (2006) used a conventional method of DON determination using immunoaffinity clean-up and RP-HPLC with DAD detection (*viz.* UV absorbance). This is a method typically supplied by the commodity testing suppliers and is therefore well validated for many matrices and gives high sensitivity, although the immunoaffinity columns (IAC) are expensive and are specific for DON, so validation is required for other trichothecenes homologues and metabolites within the sample.

Driehuis *et al.* (2008) undertook an analytical survey of MT in Dutch silage using LC-MS/MS, the gold standard in MT detection due to the capability for high sensitivity multi-residue analysis without excessive clean-up steps. A combination of these approaches was employed successfully by Döll and colleagues (2009) to achieve the sensitivity required for DON-conjugate analysis in cell culture media, although gas chromatography can also be used in equally complex matrices such as simulated gastrointestinal fluid (Avantaggiato *et al.*, 2004).

Table 1.3. Examples of DON detection and quantification protocols in animal fluids, feed components and bacterial liquid media.

Matrix and extraction details including % recovery	Separation method	Mode of detection and sensitivity	Reference
Fusarium-contaminated wheat and maize. Samples oven dried, extracted with 88:12 H_2O/ACN, immune-affinity clean-up with DONprep columns (R-Biopharm). 74% recovery	RP-HPLC (reverse phase high pressure liquid chromatography), C18, 250 mm x 3 mm, 4 µm, 40 °C, 0.5 ml / min isocratic 12% ACN v/v, 50 µl injection	Diode array detection (DAD), LOD; 30 µg/kg DM	Seeling *et al.* (2006)
Maize and whole crop wheat silage. Sample oven dried, powdered, mixed with 80% ACN, diluted and filtered. 73-111% recovery.	RP-HPLC, C18 150 mm x 3.2 mm, 5 µm, binary gradient of 0.1% formic acid in H_2O/ACN, 0.3 ml/min, 20 µl injection	Tandem mass spectrometry (MS/MS), triple quadrupole, LOQ; 250 µg/kg DM	Driehuis *et al.* (2008)
Dialysate fluid from dynamic GI absorption model extracted with ACN, dried under N_2, cleaned up in a Mycosep® column, derivatised using Tri-Sil TBT, partitioned with hexane and dried with Na_2SO_4	Gas chromatography, RTX-5 capillary column, 30 m x 0.32 mm, 0.25 mm film thickness, detector temperature; 310 °C	Electron capture detection (ECD). No LOQ given as all relative peak areas.	Avantaggiato *et al.* (2004)
Cellular and tissue growth media extracted twice with ethyl acetate, then passed through disposable chem. Elute columns and DON test immunoaffinity columns. 88% recovery of which 54% was found conjugated with glucuronic acid and released following a 24 h 37 °C pH 5.5 incubation with β-glucuronidase prior to extracting.	Liquid chromatography-HPLC C18 column; 100 mmx2.1 mm, 3 µm, binary gradient with 0.31 mM ammonium acetate in H_2O (pH 7.4) and ACN.	Electron spray ionization tandem mass spectrometry with external calibration, LOD; 1.6 µg/l	Döll *et al.* (2009)
Pig's urine and maize extracted with multifunctional MycoSep column. 64-102% recovery for all compounds in both matrices.	HPLC column; Polar-RP C18 column. H_2O:ACN:MeOH isocratic with 1 ml/min flow rate.	Atmospheric pressure chemical ionisation mass spectrometry. LOD; 25-150 ng/g	Razzazi-Fazeli *et al.* (2003)

1.4 Preventative measures and post-harvest treatment strategies for reduction of DON levels in cereal crops

Levels of MTs for both plant pathogenic and spoilage moulds are directly linked to the amount of toxigenic mould present which is related to whether optimal conditions conducive to spore germination, mycelial growth and reproduction are present and whether plant, microbial and climatic antagonistic stimuli induce MT production. The effect of climate change on mould ecophysiology and MT production has also been brought to the fore (Magan *et al.*, 2011).

Toxigenic plant pathogens such as *Fusarium* spp. can be kept in check with topical application of fungicides, employing good farming practices such as crop rotation, tilling of the soil and growing of resistant cultivars (may include future use of genetic manipulation technology) (Beyer *et al.*, 2006, Edwards, 2004). However, wet weather during certain stages of grain growth is a greater correlational factor concerning DON-contamination (Langseth and Elen, 1997).

Daliè and co-workers (2010) found significant inhibition of *Fusarium* sp. growth by *Pediococcus pentosaceus*, a lactic acid bacteria (LAB) isolated from the maize, therefore illustrating that an innovative prevention strategy may be to use LAB in place of fungicides (and illustrating that by employing the inhibitive properties of bacteria already in competition with *Fusarium* sp. it may be possible to reduce MT levels). Use of LAB as bio-control agents (BCAs) has been also studied in the context of bread spoilage by Gerez *et al.* (2009) who found lactic, acetic and phenyl-lactic acid to play a vital role in inhibiting both mycelia and conidia spore germination. The results of this study relate well to the need for inhibition of toxigenic spoilage moulds during grain storage, such as *Aspergillus* sp. and the production of ochratoxin A (Böhm *et al.*, 2000). This can be extended to LAB in fermented feeds where *de novo* production of DON post-harvest needs to be prevented in addition to inhibition of other toxigenic mould species.

Bacteria are a particularly diverse group of organisms in terms of substrate metabolism, and it is theoretically plausible that any of a wide range of natural and xenobiotic pollutants could be targeted with the use of a metabolically capable bacterial strain. Ideally the metabolism of toxin yields simple, innocuous precursors or at the very least, a toxin with lowered toxicity produced by irreversible biotransformation. LAB have already

been shown to reduce the toxicity of dietary heterocyclic aromatic amines (Orrhage *et al.*, 1994), benzo[a]pyrene and heavy metals with dietary inclusion of LAB in humans correlating with reduced incidences of colon cancer by a range of suggested mechanisms (Rafter, 2002, Bolognani *et al.*, 1997). The two main detoxification mechanisms focused on in the literature concerning LAB are toxin binding and enzymatic biotransformation.

1.4.1 The binding of mycotoxins

The binding of MTs to clay, activated charcoal and microbial polysaccharide membrane including LAB has been very effective for the so called "absorbable" MTs that includes AFB_1 (Hernandez-Mendoza *et al.*, 2009). DON has been shown to bind *in vitro* to certain LAB strains (El-Nezami *et al.*, 1998) and *Bacillus* sp. (Cheng *et al.*, 2010), yet the tenacity of the interaction following GI transit is contested by Avantaggiato and co-workers (2004), who found many DON binding agents to be grossly inadequate when tested in an *in vitro* gastrointestinal model, with the majority of any binding being reversed. Importantly, *in vivo* binding studies are notorious for showing huge variation in zootechnical parameters. Binders are often non-specific and whilst they are possibly functional, they bind essential micronutrients such as vitamins; thus reducing animal productivity but also quenching the binding sites of the binder and consequently reducing the capacity for MT removal (Tomašević-Čanović *et al.*, 2000). Binders can be very matrix/animal specific suggesting innate binding or diet effects may be confounding factors *in vivo*. It must be noted that many of the *in vitro* binding studies so far attempted have shown a degree of binding takes place with most materials, yet few have compared such activity to that of indigestible dietary elements such as insoluble fibre, or even the likely contaminated matrix used as feeds; i.e. maize (Lemke *et al.*, 2001).

1.4.2 Bacterial biotransformation of DON

The first suggestion of the possibility of trichothecene degradation was elucidated as far back as the 1960's by Horváth and Varga (1961) whilst investigating such toxins as possible antibiotics, however the treatment and mechanism was not indicated. The first known metabolite of DON biodegradation, deepoxy-DON (DOM-1), was initially characterised by Yoshikawa and co-workers (1983) in rat urine and later found to be produced following an *in vitro* rumen incubation, in cow's milk (Cote *et al.*, 1986, Yoshizawa *et al.*, 1986) and pigs urine (Razzazi-Fazeli *et al.*, 2003). This forged the concept that bacterial biotransformation was indeed possible and this was further confirmed by work with chicken and pig intestinal contents *viz.* microflora (He *et al.*, 1992, Sundstøl Eriksen *et al.*, 2004) .
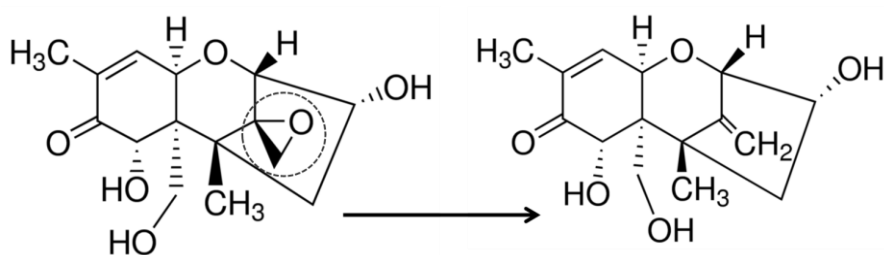
Figure 1. 2 The structure of DON (right) and DOM-1 (left) with the epoxide moiety highlighted (Sigma-Aldrich)

The epoxide structure is intrinsically linked to the toxicity of DON, and exchanging it for a double bonded CH_2 dramatically reduces its toxicity (DOM-1 was 500X less toxic in exposed mouse fibroblast 3T3 cells, (Sundstøl Eriksen *et al.*, 2004)). This is now known to be possible using bacterial epoxidases derived from two commercially developed detoxifying bacteria that are sold to perform this task *in vivo*. These strains are DSM-11798 (*Coriobacteriaceae* sp., previously *Eubacterium* sp.) and DSM-11799 (*Enterococcus casseliflavus*) which are patented by Biomin® as BBSH-797 and isolated from cow rumen

(Fuchs *et al.*, 2002). Such anaerobic strains are better incorporated into the diet for biotransformation in the rumen or GI tract where anaerobic conditions and optimal temperature allow such processes to occur (Awad *et al.*, 2006). These strict requirements for growth preclude the use of these detoxifying agents to treat contaminated grain in a fermented feed detoxification strategy, although finding one suitable to these conditions would be very valuable indeed, as the chief danger of using additives that only function during digestion is at times there may be variations in gut transit time limiting effective detoxification. A more recently isolated soil borne bacteria, *Citrobacter freudii*, with de-epoxidase capacity under both aerobic and anaerobic conditions across a wide range of temperatures shows increased potential as a true postharvest amelioration additive as it can hypothetically be applied and reduce DON at the feed formulation stage rather than following ingestion. *C. freudii* is a facultative anaerobe and opportunistic human pathogen found in a wide range of environments from the soil to the lower intestine where it converts nitrite to nitrate during respiration (Wang *et al.*, 2000). It is therefore a surprising candidate for such an application, although as the researcher suggests the genes responsible could be inserted *in planta* for maximum effect (Islam *et al.*, 2012).

Whilst de-epoxidase has been found to be effectual for modification of the epoxide moiety, other research has found other modes of biotransformation that could be employed for a similar effect. Theison and Berger (2005) screened a bank of epoxide hydrolase organisms for the biotransformation of DON and found that the mould; *Alternaria alternata* f. sp. *lycopersici* AS27-3, converted around 50% of DON into a metabolite dissimilar to DOM-1 and more polar to DON (identified with TLC), following incubation for 6 d with the model epoxide hydrolase substrate; trans-stilbene oxide, as

the main carbon source and inducer. The transformation of epoxides by microbes is relatively complex compared to in mammals, but the provision of epoxide hydrolase enzymes is broadly the same (even though the enzyme structures are very different)(Swaving and de Bont, 1998). If an epoxide hydrolase enzyme were responsible for the biotransformation of DON, then it would be prudent to screen any potential microbial detoxification candidates for the ability to produce such an enzyme. It is worth also considering that the phenomenon of co-metabolism could come into play. Enzymes produced by the bacteria to metabolise one substrate may be partially effective at metabolising a slightly different one, hence the use of sole carbon source enrichment strain selection. Such an interaction may have little benefit for the bacteria, yet in the context of mycotoxin degradation, it could be fortuitous. Since DON contains an epoxide group, it is possible that epoxide hydrolase will be an important enzyme worthy of further investigation. Epoxide hydrolases are found in most organisms and function in preventing toxicity from dietary epoxides such as limonene-1,2-epoxide (LIMOX) (Hammock and Morisseau, 2005). LIMOX was incidentally chosen as the co-metabolism substrate in the current screening schedule.

Another biotransformation pathway elicited by Shima and co-workers (1997) using a soil bacterium of the *Agrobacterium-rhizobium* group, produced a different product to DOM-1, namely 3-keto-4-DON by a suggested enzymatic mechanism, which was one tenth as immunosuppressive as DON using a lymphocyte proliferation assay. The strain was isolated using enrichment medium where a group of bacteria is incubated long term with DON and the strain able to withstand and proliferate following fourteen serial inoculations into fresh enrichment medium (200 µg/ml DON) is identified. This end-

product was also found in a mixed culture by Völki and co-workers (2004) when screening 1285 farmyard-isolated strains. The hypothesis behind testing so many farmyard-isolated strains was the notion that even though DON is prevalent, there is no accumulation in the soil and therefore some biological process must be responsible, with bacteria the most likely entity.

One practical aspect of these biological solutions to DON contamination is that these species are unlikely to function in low pH, LAB-based fermented feeds, the main subject of investigation in the current thesis, and therefore a different approach is perhaps required to find a biotransforming bacterium which fulfils these criteria. The acid conditions generated in ensiled crimped grain (CG) or liquid feeds (LF) by production of lactic, acetic and propionic acid work well at preventing the growth of undesirable organisms such as spoilage moulds, yet the indirect role of these bacterial metabolites on the degradation of DON has not been explored in animal feeds. A study by Méndez-Albores $et\ al.$ (2007) working with AFB_1 found a 1 N citric acid treatment reduced levels by up to 86% in extruded maize beckoning the question of why other acids have not been given similar attention. Non-enzymatic hydrolysis or degradation of MT due to acidic conditions is a relatively unknown area of research, but one of especial relevance when concerning ensiled feeds. Dänicke and co-workers found DON-contaminated grain treated with sodium bisulphite and propionic acid concurrently was more effective at DON-degradation than sodium bisulphite in isolation (Dänicke $et\ al.$, 2009, Beyer $et\ al.$, 2010). This transformation required strongly acidic aqueous conditions, however, the provision of a suitable enzyme eliminates this requirement (He $et\ al.$, 2010a). This was

found to occur with an *Aspergillus* sp. which was able to increase the molecular weight of 94.4% of DON by 18.1, *viz.* hydration of the C=C (He *et al.*, 2008).

An important final point is that the EU commission currently prohibits the use of remediation agents where the level of grain contamination exceeds set limits for animal feed (Table 1.2).

"The use of such products may not result in an increase of the existing maximum or guidance levels established in the context of Directive 2002/32/EC of the European Parliament and of the Council (2), but should improve the quality of the feed for animal nutrition which is **lawfully on the market**, providing additional guarantees for the protection of animal and public health." EU regulation 386/2009, (European-Commission, 2009)

This regulation prevents the use of any agent to rectify grain which would not be permitted to feed to cattle for example, nevertheless since a large proportion of forage grain is presumably ensiled without undertaking any on-farm MT testing and there is not yet an enforcement of these regulations in silage, the justification for applying a detoxification agent to fermented feed would be for preventative purposes or following anecdotal risk awareness. The response of many feed additive companies would be that any level of contamination affects growth and therefore any reduction in levels is advantageous. On a similar theme, the biotechnology companies supplying silage additives work hard to find evidence of MT detoxification in existing fermented feed inoculants as this would be a one size fits all solution, an avenue explored in Chapter 2. Undertaking detoxification processes within a fermented feed silo can also be seen to complement detoxification capability within the animal; in fact the silo could be seen as an extension to the rumen in some respects.

1.5 Sources of potential detoxifying bacteria

Many organisms are exposed to MTs, which can be traced to the offensive/defensive efforts of potential pathogens, hosts or competitors. Evidence suggests that the most MT-susceptible organisms are not always the host fungus, an interesting example of this being that Rainbow trout are the most sensitive organisms to the carcinogenic effect of AFB$_1$ (Bailey *et al.*, 1988). One hypothesis is that the least susceptible organism is the one which habitually comes into contact with the toxigenic mould, or even consumes it directly for sustenance. In this case the organisms survival depends on a suite of biotransformation enzymes encompassing conjugative, oxidative, reductive and hydrolytic mechanisms (Shen and Dowd, 1991), or the instrumental involvement of symbiotic and commensal gut bacteria. The properties of such bacteria can potentially be exploited for MT remediation and the quest for the most efficacious strains is progressing quickly (Karlovsky, 2011). Some potential sources of such bacteria are explored below.

1.5.1 Production animals: monogastrics, ruminants and chickens

Monogastric organisms include pigs, horses and humans. Plant based MTs are absorbed into the bloodstream in the small intestine for metabolism and excretion by the liver. The large intestine is where the majority of bacterial interactions occur, including the metabolism of MT (Pestka, 2007). Pigs have been found to have a small quantity of the far less toxic DON metabolite; deepoxylated-DON (DOM-1), presumably accomplished by such lower intestinal bacteria (Kollarczik *et al.*, 1994). The low susceptibility of ruminants to DON was found to be largely due to bacterial biotransformation in the rumen of DON to DOM-1 (Yoshizawa *et al.*, 1986, Yoshizawa *et al.*, 1983), hence this is a good place to source degradation bacteria. However, the strongly anaerobic conditions in the rumen mean that such fastidious bacteria are difficult to use in fermented animal feeds where

microaerophillic bacteria are more suited. Young *et al.* (2007) found the contents of chicken digesta to have the potential for biotransformation of DON to 1-DOM, with the two strains testing positive for this ability (LS100, 5S3) found to also transform other trichothecenes as well, albeit not producing the same species of product. The non-acetylated trichothecenes such as DON, nivalenol and verrucarol were transformed to their de-epoxy metabolites and the acetylated trichothecenes such as 3-acetyl DON, 15-acetyl DON and fusarenon X transformed to their deacetylated products. Yunus and co-workers (2010) found low de-epoxidase ability in acutely DON exposed chickens, but overall found only 0.04% of the fed DON was found in the plasma after 1h suggesting such a metabolism may be insignificant due to the very low uptake in the gut, however with a chronic exposure it was found that regions of the gut such as the crop had progressively more DOM-1 with acclimation time, suggesting physiological or microbial assemblage changes were induced (Yunus *et al.*, 2012)

1.5.2 Wild birds

The effect of MTs on wild birds is a completely unexplored area and there is a general paucity of data regarding wild birds as a source of LAB in general. The current investigation will attempt to isolate LAB from granivorous birds obtained from the wild (both wild reared and birds reared in captivity and released) through shooting or natural deaths. The significance of them being wild sourced stems from them firstly habitually feeding on grain that could be contaminated with toxins (*viz.* MTs and seed antifeedant/digestion compounds) and secondly having a crop-sac where such grain is stored and is known to host significant amounts of LAB which could play a role in toxin resistance. Wild mallard ducks in the grain-producing belt of Canada obtain 90% of their

energy needs from grain during the harvest season (Clark and Sugden, 1990), although they showed no sign of toxicity or feed refusal when fed highly contaminated wheat (naturally 5-8 μg/g) in a 2 wk experimental study (Boston *et al.*, 1996). One advantage of isolating LAB from birds is that they would be immediately compatible with poultry in terms of temperature and may have probiotic or gut assemblage shaping potential.

1.5.3 Grain-silo insects

There is generally a strong link between insect infestation of growing cereals and stored commodities and toxigenic mould incidence leading to presence of MT. Insects damage the grains whilst feeding leaving a favourable situation for moulds to colonise and in some cases actually act as spreading vectors for mould by carrying the spores and sporangiospores on the integument or as contents of the gut, for some insects seek mould as primary forage. An example of mould consumers are the Psorcids, which feed on a range of moulds which include *Aspergillus flavus* and *Fusarium* spp. (Kalinovic *et al.*, 2006) and have therefore been implicated in the increased presence of MT. Since Psorcids consume on toxigenic moulds, it is conceivable that they possess mechanisms for detoxification, which could include a microflora element. Since they also feed on bacterial biofilms there is potential for picking up candidate detoxifying bacteria. Dowd (1992) proposed that dried fruit beetle (*Carpophilus hemipterus*) which feeds primarily on mould was able to hydrolyse a model trichothecene, although it was not found that a microbial enzyme was responsible. The author states that such a mechanism is likely derived from the insect's need for resistance against plant allelochemicals and secondary metabolites.

1.5.4 Soil, cereal phyllosphere, silage and moulds

Volki and co-authors (2004) hypothesised that since DON is widespread in arable agriculture and does not seem to accumulate in the soils, the most potential source of bacterial isolates capable of toxin metabolism theoretically lies within the soil, grains or insects. However, the only bacterial strain that seemed to degrade DON to 3-keto-DON was isolated from pond water. More recent farm platform work presented by Wettstein *et al.* (WMF, 2014) has confirmed that DON does not accumulate but actually leaches from the soil into neighbouring water courses; hence this pond-isolate is still fairly relevant. Shima *et al.* (1997) did however find a soil bacterium able to metabolise DON to the same metabolite following enrichment culture using DON as a sole source of carbon over an extended anaerobic incubation period.

Plants have innate mechanisms to combat the effects of DON, such as glucosidic conjugation and phenolic acid production (Engelharclt *et al.*, 2002, Poppenberger *et al.*, 2003). The role of epiphytic commensal bacteria on the ecological interaction between *Fusarium* sp. and the plant and whether these bacteria aid in MT removal is an exciting avenue of research starting to yield interesting results. Daliè *et al.* (2010) for example, isolated a *Pediococcus pentosaceus* (L006) from a maize leaf with novel antifungal properties that could be used as a bio-control agent (BCA) against toxigenic *Fusarium* sp. in growing crops, therefore reducing MTs prophylactically. It can be envisaged that such bacteria, whilst commensal in growing plants, are opportunistic decomposers of damaged plant tissue and the pioneering inocula for silage fermentation, hence would be exposed to DON in this capacity (chapters 3, 4, 5). Commercially available silage inoculants were all presumably isolated from naturally fermented material (and therefore would have existed in the living plant) and screened for the ability to effectively preserve ensiled

material from deleterious organisms. The possibility of these commonly used strains also having the capacity for DON removal in fermented feeds forms the basis for chapters 2 and 3.

The concept of reducing DON in naturally contaminated grain using a DON-degrading bacterium derived from the phyllosphere of the wheat or barley itself has been recently explored by Ito and co-workers who isolated a *Marmoricola* sp. from the heads of wheat and applied it to DON-contaminated material, yielding a 66% decrease in DON (2012). This illustrates how naturally occurring microbial MT biodegradation pathways could be employed both for reduction of FHB effects in planta and in harvested commodities (Ito *et al.*, 2013). Whilst this is extremely encouraging, the current study would require that an epiphytic isolate not only has the DON-degradation ability but also has the potential for growth and amelioration of DON within a fermented feed scenario.

As a side point, the fact that DON does not affect the mould producing the toxin is evidence that non-activation and possibly deactivation is possible with specific enzymes present. Morisseau *et al.* (1999) found high levels of epoxide hydrolase in *Alternaria* sp. concomitant with levels of AAL toxin production (sphingoline homologue) indicating a molecular synthesis application that could be used for exogenous compounds too. This avenue is beyond the scope of the present study.

1.6 Fermented animal feeds: an opportunity to remove mycotoxins and increase feed value and safety

1.7 Screening for mycotoxin bioremediation properties, including evaluation of biotransformation and binding efficacy in a variety of conditions

1.7.1 Screening techniques

The main techniques for screening for a specific metabolic trait range from; using the pure form of the specific compound in question and measuring transformation spectrophotometrically, using chromatography or immunodetection; to using the compound as an sole carbon source enrichment component where bacteria able to metabolise the compound/structure are able to reproduce and be detected as growing colonies in minimal media (Teniola *et al.*, 2005). Such an approach is also used for structural homologues of the compound/toxin in question, where due to cost, availability or toxicity, the MT is replaced by a compound sharing structural moieties that if metabolically modified may indicate the ability to metabolise the MT by proxy, and as such is a valid first screen approach.

Whereas Theison and Berger (2005) used DON surrogate homologues such as styrene oxide as a sole carbon source to screen for epoxide metabolism properties in a range of microbes, other workers have used the same molecule when screening for epoxide hydrolase activity in marine bacteria (Hwang *et al.*, 2008). Such an epoxide hydrolase enzyme is also responsible for transforming limonene-1,2-epoxide into limonene-1,2-diol, and since this is a commercially available flavouring with low toxicity, (Arand *et al.*, 2003) it is chosen as the sole carbon source in the current investigation.

1.7.2 Procedures for evaluating remediation efficacy

Obviously the best way to determine if DON has been degraded would be to analyse the spiked media/grain matrix prior to and after LAB inoculation. ELISA or HPLC methods can be utilised for such a purpose, however since the derivatives or metabolites of DON may share a degree of toxicity with the parent molecule it is important to verify the safety of the fermented product (Sundstøl Eriksen *et al.*, 2004). Past studies have utilised cell lines and sterile filtered MT growth medium to evaluate cytotoxic, genotoxic or carcinogenic responses, and in doing so have highlighted the need to characterise not only the efficacy of MT removal but also toxicity of the finished product (Kollarczik *et al.*, 1994, Mokoena *et al.*, 2006). This should be extended to the cell membrane MT-binding phenomenon frequently found in the literature, see tables 1.4 and 1.5 (Jin *et al.*, 2015). Should the supernatant of the medium be extracted, then it would be conceivable that the concentration of MT may have reduced from time zero, however if we were able to fully extract the pellet/ matrix then the MT may be shown to concentrate in to this fraction. The adsorption properties between the cell membrane/ matrix and MT dictate the efficiency of extraction and recovery leading to an underestimation in terms of concentration, but could be a valuable mechanism worthy of investigation. Furthermore and more crucially, should the MT be bound and toxicologically innocuous during animal feed digestion and GI transit, without affecting the nutritive content of the liquid animal feed, then a beneficial trait is present (this binding phenomenon forms the basis of physical and biological remediation methods discussed in section 1.4). Should the efficacy of binding be reduced following changes in pH, ionic strength (bile salts) or presence of extracellular enzymes in the stomach, then it is conceivable that toxicological status of the feed may resort back to previous levels and thus removing the remediation

potential of the binder/microbe (Avantaggiato *et al.*, 2004). In order to verify whether

binding is a possible characteristic to utilise it is necessary to carry out not only

simulations of gut conditions following binding, but also evaluate the strength of

interaction by extracting with a range of solvents/heat and determining if both viable and

non-viable cell fractions share the beneficial characteristic (Haskard *et al.*, 2000, El-

Nezami *et al.*, 2002).

Table 1.4. Examples of different methods of determining the efficacy of AFB_1 inactivation in a range of bacteria-inoculated liquid media/feeds

Media, MT and treatment	Proposed mechanism of MT inactivation	Evaluation of remediation value	Reference
Maize meal (58.3% H_2O), AFB_1, *Streptococcus lactis, Lactobacillus delbrueckii* incubation	Not established	Cytotoxicity of LF extracts evaluated in human esophageal cell line using the MTT assay. Shown that LAB reduced toxicity of extract.	Mokoena *et al.* (2006)
MRS broth, AFB_1, strains of *Lactobacillus casei* incubation	Binding	Washing steps and exposure to bile salts to simulate digestion showed a 0.6-9.2% release was possible.	Hernandez-Mendoza *et al.* (2009)
Dry AFB_1 contaminated maize, heat and 1 N citric acid treatment (3 ml per g of maize)	Chemical inactivation	End product shown to have reduced genotoxicity in the Ames test and was innocuous as duckling feed.	Méndez-Albores, Del Río-García et al (2009)

Table 1. 5 Examples of different methods of determining the efficacy of DON inactivation in a range of treatments and media

Media and treatment	Proposed mechanism of MT inactivation	Evaluation of remediation value	Reference
PBS for *in vitro* activated charcoal binding experiments and dry wheat (with activated charcoal) for GI absorption model	Binding	The binding affinity was analysed by determining the percentage of bound DON after *in vitro* incubation, then by determining the percentage of DON absorbed in the simulated small intestine (Jejunal/Ileum) following addition of activated charcoal. With a 2% inclusion rate, the absorption of DON was almost halved	Avantaggiato *et al.* (2004)
Viable and non-viable bacterial cells assessed for binding of trichothecenes including DON at 20 µg/ml in PBS for 1 h at 37 °C	Binding to cell membrane (no degradation products found with GC-MS)	4 out of 7 trichothecenes were bound *in vitro* (incl. DON) with binding of 18-93% found for *L. rhamnosus GG, L. rhamnosus LC-705* and *Propionibacterium freudenreichi ssp. Shermanii. In vitro* results need to be supported by *in vivo* data, as the binding complex may not be strong enough to pass through the GI tract.	El-Nezami *et al.* (2002)
Detoxification of DON in wheat or maize served as a realistic model for use in contaminated animal feeds for two strains of *Bacillus* sp.	59 probiotic strains incubated with 1000 mg/L DON and degree of degradation measured using indirect competitive ELISA. Parallel study looked at the interference of MRS medium, with neutralisation and 32-fold dilution yielding better results.	Due to the measurement being by ELISA, confirmation that the change is non-reversible is speculation. Had the metabolites been measured with chromatography, more information would be available about the de-toxification mechanism, although the spent cell supernatant held the important element responsible which was heat labile, hence was likely to be an enzyme, however the authors mention that another agent may be also important in DON interaction. (This paper states *B. subtilis* and *B. licheniformis* as decreasing DON by 80 and 14-44% respectively, yet a study by Dänicke *et al.* (2010) found the addition of these strains *in vivo* to have no effect.)	Cheng *et al.* (2010)

1.8 The role of D3G when considering fermented feeds

As previously mentioned DON is a disease virulence factor enabling swift colonisation of crops; however some cultivars are able to reduce this impact by conjugating DON to a glucoside residue and locking it out of toxic contention as DON-3-glucoside (D3G). This phenomenon actually makes consuming the grain potentially more risky as D3G is

converted back to its toxic precursor during gastro-intestinal (GI) enzymatic transit and this form of DON may not be factored in a grain analysis strategy (Nagl *et al.*, 2012).

D3G or "masked" DON is a relatively recent discovery that has sent shockwaves through the food and feed testing industries as it was thought levels of DON could be accurately determined by well validated testing methods in a wide range of commodities and products allowing risk assessments to be written and maximum contamination levels to be set (Klötzel *et al.*, 2005, EFSA, 2013). These maximum contamination levels in a range of cereal products are calculated to prevent acute and chronic toxicity and associated drops in productivity in production animals. Although they have a margin of safety, if a proportion of DON was masked from the analysis in D3G form, the actual level of DON exposure in the animals gut may be significantly higher following cleaving of the glucoside residue (Rasmussen *et al.*, 2012). It is therefore crucial that it is included in any future DON determination in cereal based animal feeds and consideration must also be made to D3G stability in fermented feeds such as CG silage and LF, especially in feed for monogastrics. It would also be interesting to determine whether D3G is biotransformed by LAB *in vitro* or within the silo environment to DON or even whether the presence of the glucoside residue facilitates further DON degradation. It would also be prudent to determine whether the process of ensiling and microbial growth plays a role in increasing the unlocking of compartmentalised DON/D3G into a more readily detectable and therefore bioavailable form in terms of animal exposure and toxicity. Simsek and co-workers (2012) investigated the fate of DON and D3G In wheat during the processing from whole grain to flour, fermentation, dough and baked bread and discovered that a large proportion of both toxins were locked up in the husk material, or attached to

kernel-borne proteins and only measurable following protease treatment. Interestingly,

When determining the levels of measurable DON/D3G during the production of beer,

Lancova *et al.* (2008) found levels of DON and D3G to increase during the malting phase

and levels of D3G to increase further during the brewing phase. This further illustrates

how levels of DON/D3G may fluctuate in fermented feeds as microbial metabolism takes

place and should be investigated.

Although the D3G form may pass further through the gut than DON before being

toxicologically active the exact timing and efficiency of this digestion is not currently

known, although in rats it was shown that in equimolar exposures a larger proportion of

DON was found in the faeces for those exposed with D3G indicating that absorption was

reduced when a glucoside was present (Nagl *et al.*, 2012) and concentrations of

glucuronidised DON for urinary excretion in pigs was reduced 2 fold when exposed as

D3G suggesting bioavailability was also reduced (Nagl *et al.*, 2014). D3G has been shown

to possess different biochemical properties to DON including; a difference in solubility

and chromatographic separation (Berthiller *et al.*, 2005).

1.9 Toxicity of DON in monogastrics

Even though the primary role of DON is to act as a virulence factor in plants, the most

sensitive organisms to this toxin are mammals, specifically the monogastrics such as

humans and pigs (Table 1.6). In pigs, symptoms of acute DON ingestion range from

emesis and abdominal stress to intestinal haemorrhage, liver damage and anorexia

(Pestka, 2007). Such effects, although not life-threatening when considering the highest

likely dose as an acute exposure, do cause a severe impact on animal productivity and

welfare during chronic exposure (Dänicke *et al.*, 2006). This is a major consideration

Table 1.6 The *in vitro* and *in vivo* toxicity profile of DON in monogastrics

Organism/Cell type	DON concentration and experimental detail	Principle symptoms and effects	Reference
Primary isolated pig hepatocytes and Kupffer macrophages	0.5-16000 nM exposed to tissue cultures with LPS (lipopolysaccharide) co-exposure. Disruption of protein synthesis determined by H^3-leucine incorporation and albumin secretion. Disruption of metabolism determined by MTT assay. Cellular membrane integrity determined by neutral red uptake assay. Determination of glucuronidation (hepatocyte conjugation) factor by use of DON/Deepoxy DON detection before and after incubation with β-glucuronidase.	A 2-4 µM DON exposure resulted in a significant decrease in neutral red uptake, but metabolism was unaffected up to 16 µM. Reduced albumin secretion by both cell types (39-47% of normal) with LPS decreasing the effect on secretion, but increasing the effect on cellular protein synthesis. Glucuronidation rate decreased with increasing levels of DON.	Döll *et al.* (2009)
Caco-2 (human colon cancer cell line)	Degree of protein synthesis inhibition determined by H^3 leucine incorporation and scintillation. Trans-well membranes seeded with cells were exposed on one side to DON (50-5000 ng/ml) and H^3-mannitol diffusion measured by scintillation. Tight junction gene synthesis activity also measured.	DON inhibits the expression of key regulatory and cell maintenance proteins (tight junctions etc.) leading to an increase in membrane permeability	De Walle *et al.* (2010)
Swiss mouse fibroblasts (3T3)	Cytotoxicity of de-epoxidated DON measured using the BrdU incorporation assay. De-epoxidation accomplished using pig microflora and purified fractions collected using HPLC.	De-epoxy DON (DOM-1) found to be 52 times less toxic than DON in terms of concentration needed for 50% inhibition of DNA synthesis	Sundstøl Eriksen *et al.* (2004)
Pigs	Pigs fed chronically with DON-spiked feed for chronic experiment and injected with acute DON levels using an *in situ* catheter. This catheter for chronic and acute exposures was also utilised for administering/ flooding the body with $^3H_6$-phenylalanine, which allowed protein synthesis inhibition in each tissue following sacrifice to be determined using GC-MS.	Protein synthesis was significantly reduced in the kidneys, spleen and ileum of exposed pigs, with the chronically exposed (4 weeks) showing greater effects, due perhaps to the differing degree of glucuronidation between the two treatments.	Dänicke *et al.* (2006)
Pigs	Pigs fed DON-contaminated feed (3.5-4.4 mg/kg) Monocyte derived dendritic cells were isolated and either tested once more with DON, or left untreated.	Dietary exposure of DON led to a reduced endocytosis of FITC-dextran in the presence of DON and LPS/TNFα by monocyte derived dendritic cells, but did not stimulate a T-cell response. Flow cytometry indicated maturation and vitality of monocyte derived dendritic cells was reduced with *in vitro* DON dosing of control diet.	Bimczok *et al.* (2007)

when formulating compound and LF for pigs, and the strict EU limits (Table 1.1) aid in reducing DON toxicity (in addition, once incorporated into LF there is a further possibility of reducing toxicity to even lower levels by inoculating with DON-detoxifying LAB (Shetty and Jespersen, 2006), Chapter 2).

The mechanism of DON-toxicity is inhibition of protein synthesis by interacting with the 60S ribosomal subunit within eukaryotic cells and preventing initiation and elongation of polypeptides (Carter and Cannon, 1977). This response can also materialise as immunosuppressive and neuro-endocrine effects contributing to the burden associated with DON contaminated feed (Pestka *et al.*, 2004, Goyarts *et al.*, 2006, Bimczok *et al.*, 2007). In pigs DON is rapidly absorbed in the small intestine and ideally conjugated to glucuronides in the liver permitting swift excretion (~8 h) (Pestka, 2007, Maul *et al.*, 2014). The evidence suggests there is no bioaccumulation of DON; therefore, this transitory period is key to the liver/intestinal toxicity. Chronic exposure to DON has been shown to inhibit intestinal cell proliferation, therefore disrupting intestinal permeability which cascades to increase DON being absorbed (De Walle *et al.*, 2010, Sergent *et al.*, 2006). Dänicke *et al.* (2006) found another result of chronic exposure (this occasion using pigs *in vivo*) was the depletion of glucuronides over time, which resulted in greater damage than those acutely dosed which were able to excrete DON more effectively. Interestingly, any detoxification of DON by biotransformation, occurs in the large intestine by resident micro flora; too late to prevent hepatotoxicity in pigs, but it does illustrate the huge difference in DON sensitivity when compared with ruminants which have ruminal microflora capable of converting DON to DOM-1, a less toxic homologue, prior to absorption (Yoshizawa *et al.*, 1986).

1.10 Toxicity and performance effects of DON in ruminants

A large proportion of the total mixed ration (TMR) for dairy and beef cattle globally is made up of fermented whole crop maize and CG, of which a large proportion is contaminated with DON (Driehuis *et al.*, 2008, Adesogan *et al.*, 2003). Fortunately the ruminants are the least susceptible production animal to the effects of DON due to microbial interactions in the rumen, though this detoxification capacity may be overwhelmed or disrupted by rumen acidosis; leading to subtle yet significant decreases in animal productivity, compromised immune function and altered rumen fermentation (Table 1.7, (Seeling *et al.*, 2006, Korosteleva *et al.*, 2009, Keese *et al.*, 2008)). By evaluating these biomarkers of effect and exposure the No Observed Adverse Effect Level (NOAEL) can challenge the guidance contamination limits outlined in the EU regulations, which are currently 5 mg/kg (12% moisture) in barley/wheat fed as principle ration. Intervention strategies should be considered should the DON content of the pre/post-harvest grain surpass the NOAEL, especially concerning pre-ruminant or transition calves which do not possess full ability to metabolise DON within the rumen (Fink-Gremmels, 2008).

Winkler and co-workers (2014) found a significant correlation between ingestion of DON-contaminated maize and concentration of DOM-1 in the plasma of 13 week-treated dairy cows with up to 10% measured as DON, suggesting ruminal de-epoxidation is not definitive and a degree of liver exposure is possible before glucuronidation-based excretion occurs. However, the Holstein cows in this study showed no change in milk production or weight gain. The effect of a mixture of DON and Zearalenone (ZEA) on ruminant acidosis was studied by Keese *et al.* (2008) who found the inclusion of infected triticale (wheat/rye hybrid) in the TMR resulted in a shift in rumen fermentation products

caused by either a modification in the microbial community and/or a physico-chemical change in the grain itself. The problem exists when such studies work with binary exposures rather than individual MTs, as it can be difficult to make firm conclusions on either component. However, MTs naturally co-occur in grain and silage (Eckard *et al.*, 2011), therefore realistic mixture exposures are justified (Grenier and Oswald, 2011) (see Chapter 6). A combination of DON and ZEA was actually implemented in the deaths of several dairy cattle in Poland following confirmation of these MTs in the serum of similarly fed cows and post-mortem analyses (Marczuk *et al.*, 2012). This is more an aetiological approach following a catastrophic mycotoxicotic incident, hence the experimental design is somewhat departed from other studies, although the researchers found indirect evidence of immunosuppression when analysing blood cell morphology and biochemical parameters.

Table 1.7 **The effects on ruminant performance and health following DON exposure.**

Ruminant details	DON concentration and experimental detail	Biomarkers of exposure and effect	Reference
Holstein dairy cows	Up to a max of 5.2 mg DON / kg DM in mixed ration made with *Fusarium* infected maize (i.e. the EU guidance value). Cows treated for 13 wk.	Levels of DON and DOM-1 in the blood plasma were closely correlated to DON in feed of which 90% was DOM-1. No adverse effects on animal growth, milk production or milk composition.	Winkler *et al.* (2014)
Holstein Dairy cows	Cows fed TMR with 5 mg DON and 113 mg ZEA / kg DM for 1 period of 11 wk, then 4.6 mg DON and 73 mg ZEA /kg for another 18 wk. The DON/ZEA content was from *Fusarium* contaminated Triticale.	Ruminal fermentation patterns and acidosis severity measured in ruminal fluid. Increase in acidosis with triticale inclusion, due to either MT effects on rumen microbes or matrix modification effects of *Fusarium* on the triticale concentrate.	Keese *et al.* (2008)
Dairy cows	TMR consisting of maize silage, cereal grains and haylage fed *ad libitum*. No MT analysis. Experimental grouping undertaken (x3) on the basis of serum DON/ZEA content following mortalities.	Aetiological study links serum levels of DON and ZEA to immunosuppression, acute immune response and death. Haematological and biochemical parameters measured in all cows and interrogated for correlation with DON/ZEA-positive groups.	Marczuk *et al.* (2012)

1.11 Toxicity profile of DON in combination with other mycotoxins and potentially with plant derived toxins such as retrorsine.

DON is an extremely effective cytotoxin that resides in many harvested cereal crops at levels that induce deleterious effects. However, DON is one of many microbial and plant toxins that could be present in harvested grain, both pre- and post-harvest. The interaction between these toxins on a tissue and cellular level in humans and production animals needs to be considered as part of commodity risk assessment and management (Verstraete, 2013).

To date there has been a small number in research articles on the co-exposure of different MTs (Speijers and Speijers, 2004, Alassane-Kpembi *et al.*, 2013, Kouadio *et al.*, 2007, Lei *et al.*, 2013, Prosperini *et al.*, 2014, Tammer *et al.*, 2007, Wan *et al.*, 2013) and a complete lack of papers associated with MTs and plant toxins, even though modern analytical methods now permit multi-toxin identification in a range of harvested materials (Berthiller *et al.*, 2007).

Assessing toxicological interaction between toxins is not a straightforward task, as it quickly becomes a highly logistical exercise as the multitude of toxin combinations and range of doses required leads to large scale experiments and synthesis of complex data that requires robust statistical interrogation (Table 1.7). Kouadio and co-workers (2007) investigated the interaction of the *Fusarium* produced MTs; DON, zearalenone (ZEA) and Fumonisin using a select range of doses aimed to simulate that likely encompassed by intestinal cells *in vivo* by consumption of contaminated grain (Table 1.8). Human-derived Caco-2 intestinal cells were exposed to binary and tertiary combinations of MTs and a suite of assays were employed to diagnose the interactive toxicological relationships between co-occurring MTs, especially those of a synergistic, potentiating or antagonistic

nature. Synergistic impacts were seen regarding lipid peroxidation and additive effects with cell viability measured using lysosomal membrane integrity, both endpoints linked to oxidative damage. Antagonistic responses were found when considering inhibition of DNA synthesis and DNA fragmentation when binary and tertiary exposures were evaluated illustrating the complexity of multifactorial analyses.

Table 1.8. Examples of studies where the interactive cellular toxicity of DON with other _Fusarium_-mycotoxins is investigated _in vitro_.

Mixtures tested and concentrations used	Toxicity assays Employed	Method of deriving interaction and result	Reference
DON (4, 10, 20 μM), zearalenone (5, 10, 20 μM) and fumonisin B1 (10 μM). These doses chosen to reflect the likely _in vivo_ intestinal cell exposure levels following ingestion of 2-4 mg/kg contaminated grain.	Neutral red uptake/retention, DNA fragmentation and methylation, protein and DNA synthesis, malonedialdehyde production (ROS) in human derived Caco-2 cells (intestinal epithelial colon adenocarcinoma).	Binary exposure combinations of listed concentrations and tertiary exposures all at 10 μM. Comparison with control and between treatments using percentage of control data and non-parametric statistics. Below additive effects seen with DNA synthesis, synergistic with lipid peroxidation and cellular viability decreasing with DON concentration most influential.	Kouadio _et al._ (2007)
DON (0.5, 2 μM), nivalenol (0.5, 2 μM), zearalenone (10, 40 μM) and Fumonisin B1 (20, 40 μM) exposed as combinations of cytotoxic and non-cytotoxic doses (from independent dose response data)	An inscribed central composite design of exposure combinations was employed to reduce the combinations from 256 to 16 and cytotoxicity measured using the MTT assay (mitochondrial metabolism) following a 48 h exposure to primary swine intestinal cells.	Non parametric statistical comparison with the control indicated at non-cytotoxic doses only those combinations where DON was tested showed significant reductions with the mix of all four MTs having the greatest effect on viability. At cytotoxic doses, all interactive exposures gave reduced viability. All data was percentage of control.	Wan _et al._ (2013)
Independent, binary and ternary mixtures of the type-B trichothecenes DON, 3AcDON, 15AcDON, nivalenol (all 0.2-6.7 μM) and fusarenon (0.007-0.12 μM).	Caco-2 exposed to MT dissolved in DMSO for 48 h after which the MTT (mitochondrial vitality) and neutral red retention assays (lysosomal integrity) employed to derive cytotoxicity. Note. the cell culture medium contained the antibiotics Penicillin and streptomycin, toxic secondary metabolites in their own right.	Two methods used: the isobologram method where the IC_{10}, IC_{20} and IC_{50} (inhibitory concentration yielding X% cytotoxicity) values are plotted together, and an additive relationship is a straight line, and a deviation above is antagonistic, below is synergistic. Combination index takes the dose required of each individual toxin to achieve X% inhibition and divides it by the interactive response at these doses. 0.9-1.1 (additivity), <0.9 (synergism) and >1.1 (antagonism)	Alassane-Kpembi _et al._ (2013)

Wan and co-workers (2013) also investigated mixtures of *Fusarium* MTs in an intestinal primary cell line, this time derived from swine, and focussed on only one cytotoxic dose and one non-cytotoxic dose of each in a selective multifactorial approach employing the MTT assay to derive mitochondrial vitality and cellular viability. Preliminary dose response curves were constructed using the individual toxins to determine which concentrations illicit cytotoxic/non-cytotoxic responses for use in interaction experiments. DON was found to be the instrumental component when in combination at non-cytotoxic doses and all combinations at cytotoxic doses yielded reduced viability over the control and antagonistic responses were absent in both dose types. Due to the lack of a multifactorial dosing strategy, there is no interaction measured between both cytotoxic and non-cytotoxic doses which does reduce the amount of treatments considerably (from 256 to 16), but at the expense of interpreting a true interactive dose response.

Alassane-Kpembi *et al.* (2013) recently used an isobologram approach where cytotoxicity responses of intestinal cells exposed *in vitro* were plotted as unary and binary exposures and the interaction term was calculated. This is perhaps the most effective approach to the problem of mixtures, however it only works when the toxins tested target the same cellular components to yield a linear dose-response.

What is apparent with these studies is that toxin mixtures have the potential to induce highly deleterious effects *in vivo,* often by complex interactions*,* and consequently more work is necessary to deduce risk factors associated with multiple-toxin contaminated food and feed. There is a lack of research in this particularly relevant area of feed and food safety (Grenier and Oswald, 2011), with the consensus seemingly being that the research community has a sufficient grasp of the toxins individually but does not wish to

commit to the challenge of toxin mixtures, unless epidemiological data already alludes to a valid interactive toxicity hypothesis, *i.e.* the apparent relationship between chronic levels of AFB_1 and Fumonisin B_1 in mouldy maize in China and the incidences of human oesophageal cancer (Chu and Li, 1994), with a strong synergistic relationship between these co-occurring MTs confirmed in rats (Gelderblom *et al.*, 2002a).

The current academic focus is on mixtures of MTs, however there are a plethora of potential field-borne natural toxins associated with weed infested cereal crops that can be a significant source of human and livestock toxicosis that should be considered as part of the general hazard assessment of toxin mixtures in cereal crops. Of particular interest is the group of 200 toxins belonging to the pyrrolizidine alkaloid group (PA) that are produced by around 300 plants worldwide including plants of Senecioneae spp. (Asteraceae family) such as *Senecio jacobea*; the common European Ragwort, or one of the more potent tropical relatives including *S. inaequidens* which was responsible for the acute deaths of several cows in South Africa (Dimande *et al.*, 2007). Both of these plants have several PAs present acting as anti-feedants, although one particularly potent PA common to both species is retrorsine (RET) and is chosen as representative of hepatotoxic Senecioneae PAs in general. Due to both DON and RET exerting significant toxicity in the liver and having the potential for co-harvest, they form the subject of a preliminary interactive toxicity study in Chapter 6.

Following consumption, PAs are metabolised in the liver by the same activation enzymes instrumental to AFB_1 genotoxicity, the cytochrome P450 monooxygenases, specifically CYP3A which yield an especially DNA and protein reactive pyrrole intermediate (DHP) (Kolars *et al.*, 1994). This manifests as DNA/protein adducts and DNA-DNA/DNA-protein

crosslinks that lead to genotoxicity and tumorigenecity, mitotic arrest and hepatic veno-occlusive disease (VOD) where megalocytosis causes a constriction of the hepatic veins leading to cirrhosis, failure and death (Figure 1.3).
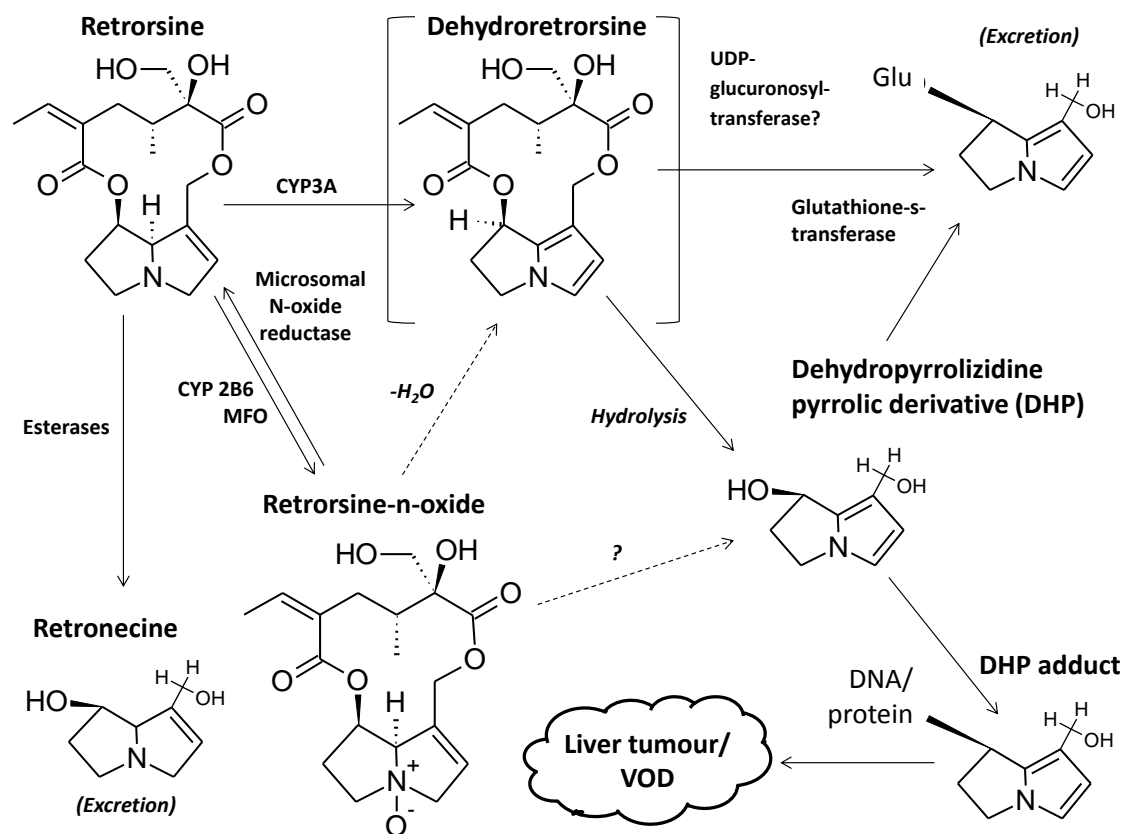
Figure 1.3 Metabolism of RET in mammals illustrating the enzymes involved in activation leading to DNA/protein-DHP adducts with the downstream possibility of mutations, mitotic arrest, veno-occlusive disease and cancer. The enzymes responsible for effective excretion are also shown. Diagram based on work published by Fu *et al.* (2002), He *et al.* (2010b) and Wang *et al.* (2005) and drawn with ACD/Chemsketch 12.01 (Advanced Chemistry Development Inc.)

Ingestion by humans and production animals is often due to co-harvesting with cereals, and poor sorting prior to processing or converting to silage (Prakash *et al.*, 1999b). Several episodes involving *Senecio* consumption are documented in Table 1.9, including the well documented study in South Africa by Dimande and co-workers (2007) which confirmed *S. inaequidens* was responsible for lethal toxicity or Seneciosis in grazing cows by administering the relevant plant extract to rats and sheep. The resulting characteristic histopathological lesions in the liver and metabolised RET measured in the liver, lungs and

urine strongly linked *S. inaequidens* to the cattle deaths. In southern Brazil Seneciosis is actually the main cause of cattle mortality, with *S. madagascariensis* and *S. brasiliensis* the species responsible (Bandarra *et al.*, 2012), both of which contain high levels of PAs that include RET. In Australia *S. lautus* (later identified as *S. madagascariensis*) *wa*s the significant weed responsible for 226 cattle deaths between 1988-1992, with pyrroles identified in the blood samples during necropsy and RET the main PA likely responsible. Another diagnostic study from S. Africa based on hospital records and necropsy evidence from 1931-41, highlights the potential for human toxicosis and lethal VOD following ingestion of bread made with PA-contaminated flour (Selzer and Parker, 1951), a theme that re-occurs in Western Afghanistan with wheat and goats milk contaminated with another PA containing plant; *Heliotropium popovii* (Boraginaceae).

Table 1.9. Summary of toxicity episodes attributed to pyrrolizidine alkaloid contamination of food/feed/fodder.

Pyrrollizidine alkaloid and plant species	Episode location and health effects	Source and cause of contamination	Reference
S. inaequidens (retrorsine, *Senecio*nine, 2 unidentified)	South Africa. Several cattle mortalities with associated severe hepatic necrosis and multiple haemorrhages	Cattle grazing	Dimande *et al.* (2007)
S. madagascariensis (retrorsine, mucronatinine, florosenine, seneciverinine)	NE Australia. 226 cattle mortalities in 4 years. Deaths occurred some months after ingestion during which the cattle lost condition, had persistent diarrhoea and had dramatic changes in behaviour. Liver necropsy yielded significant lesions in common with Seneciosis and blood/liver sample extractions showed pyrroles were present	Cattle grazing	Noble *et al.* (1994)
Senecio spp. (unknown)	South Africa. 12 human cases with severe acute VOD of which 6 died (and 12 mules locally)	In all cases bread was made from locally milled wheat flour where the wheat was imperfectly winnowed	(Selzer and Parker, 1951)
Heliotropium popovii (heliotrine, lasiocarpine, jacobine)	Afghanistan. 400 human cases with 100 deaths caused by VOD and liver necrosis.	Contaminated wheat flour made into bread and to a lesser extent goat milk.	(Kakar *et al.*, 2010)

Whilst it would appear that these records are from a growing environment very much departed from European cereal cultivation, there is growing evidence that with climate change and with the spread of non-native *Senecio* spp. rife throughout the globe there could be an incident where FHB affected and PA producing plants could be co-harvested, or at least incorporated into the total mixed ration (TMR) of cattle as silage supplemented with cereal grain. For example where wheat and barley is grown in dry countries inundated with *Senecio* spp. such as South Africa, where large-scale irrigation is necessary, it has been recently shown that FHB caused by *F. graminearum* and *F. culmorum* is becoming more common, along with DON (Boutigny *et al.*, 2011). DON producing F. culmorum has been recently conformed in 40% of harvested maize kernels in one study in Southern India (Venkataramana *et al.*, 2013), an area where the PA-producing Crotalaria spp. have caused numerous cases of human mortality (Prakash *et al.,* 1999a) It will also always be the case that in areas where rural subsistence farming is common, inadequate quality control and winnowing may occur, and climatic anomalies may both encourage the growth of FHB/*Senecio* spp. and necessitate the use of contaminated grain for consumption or as animal feeds. Furthermore, it has been shown that chronic, low levels of DON in feed over an extended period of time can disrupt the normal process of xenobiotic metabolism in mice (Gouze *et al.*, 2006), leading to a potential increased susceptibility to toxins such as RET. This will be investigated using an established human liver cell line *in vitro* in Chapter 6.

1.12 Research questions to be addressed in the current investigation

Despite the vast research interest in reducing FHB and DON production in the field, there is currently no method of preventing DON from entering the feed and food chains

completely; hence strict grain testing and risk assessment regimes are necessary to reduce downstream toxicological effects in a range of consumers with differential susceptibility (Table 1.1). Grain that is either DON-positive or not tested at all due to the "farm produced feed" nature of the crop comprises significant risks to the farmer in terms of animal productivity and welfare. This warrants the development of an effective biological method of post-harvest DON detoxification within the fermented feed environment. In addition, DON rarely exists as a single contaminant in crops and many co-occurring toxins have been shown to induce toxicosis in humans and production animals, therefore to put the DON detoxification element in perspective; the toxicological impact of DON with a co-exposed crop weed derived liver toxin is explored. The main research questions to be addressed in the current investigation:

- Do LAB play a role in reducing levels of DON in fermented animal feeds?

- Do commensal LAB in wild birds have DON detoxification potential?

- Are there other bacteria associated with fermented grain feeds which could be developed for DON biodegradation, specifically in CG feeds?

- Does DON exhibit a degree of interactive toxicity following with co-exposure with other co-occurring plant derived liver toxins, and what mechanisms are responsible?

1.12.1 Crimped grain silage, fermented liquid feeds and biofuel co-product utilisation

Forage crops such as maize, barley and wheat make up a large proportion of animal feed globally of which the majority is ensiled in on-farm silos utilising naturally occurring or artificially inoculated fermentative LAB as a safe and effective method of preserving the nutritive value of the feed, whilst preventing the growth undesirable microorganisms (Elferink *et al.,* 2000). Since these crops are likely to be contaminated to varying degrees

with a range of MTs including DON, it would be valuable to know the fate and behaviour of DON within these fermented feed silos, especially if the microbial community or individual bacterial species could be aligned to any decrease in DON leading to an associated increase in feed safety and performance.

The ensiling process is divided into 4 biological phases post-harvesting. The aerobic phase involves the rapid drop in oxygen within the silo due to physical compression, the presence of facultative aerobic microorganisms and the residual respiration capacity of the plant material. Providing the pH remains high the plant material has a degree of enzymatic competency leading to autolysis of protein and carbohydrates. As anaerobic conditions advance, the main fermentation phase begins where homo- and heterofermentative LAB (Figure 1.4, 1.5) metabolise water soluble carbohydrates (WSC) to form predominantly lactic acid (plus acetic acid, ethanol) and dropping the pH dramatically from around pH 6 to 4 leading to the inhibition of carbohydrases/proteases and undesirable biodegradation microorganisms. At this pH only a few obligate heterofermentative LAB, such as *L. buchneri*, are still metabolically active and produce further amounts of acetic acid from the metabolism of lactic acid to reduce the pH further to around 3.8 and underpinning this stable phase. It is the presence of this acetic acid which minimises aerobic instability following silo opening by inhibiting aerobic microorganisms and preventing dry matter (DM) loss in the aerobic spoilage phase (McDonald *et al.*, 1991, Dunière *et al.*, 2013, Elferink *et al.*, 2000).

Fructose   ATP

ADP

Glucose   ATP

ADP

Glucose-6-phosphate

Fructose-6-phosphate   ATP

ADP

2-Glyceraldehyde-3-phosphate

$2NAD^+$

$2NADH + H^+$ 4ADP

4ATP

2-Pyruvate

$2NADH + H^+$

2NAD

H_3C OH
OH

Lactate
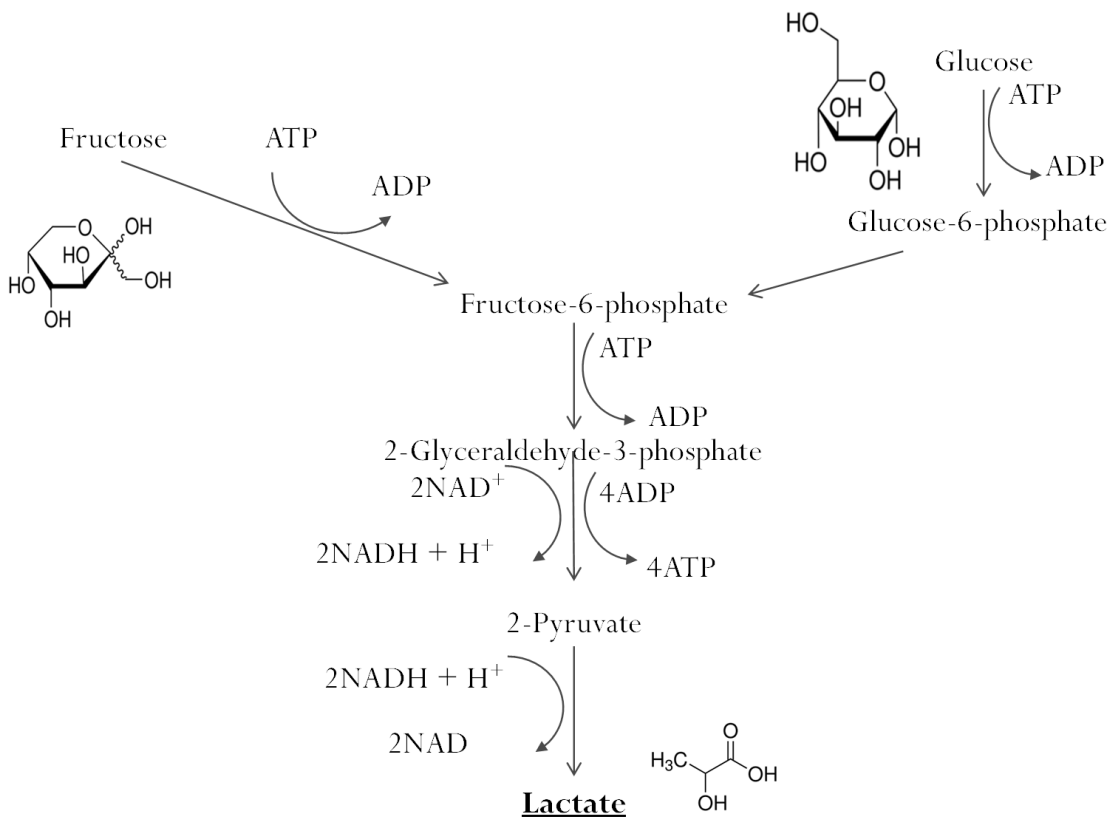
Figure 1.4. Fermentation of glucose and fructose by homofermentative LAB such as *Pediococcus acidilactici, L. plantarum** and *Streptococcus faecium* forming lactic acid. Figure redrawn from McDonald and Henderson (1991)

Glucose   ATP

ADP

Fructose   ATP

ADP

Glucose-6-phosphate   ←   Fructose-6-phosphate

$NADPH + NADP^+$

$2NADPH + H^+$ $\underline{CO_2}$

Ribulose-5-phosphate

ADP

ATP

2-Glyceraldehyde-3-phosphate   Acetyl-phosphate   **Acetate**

$2NAD^+$ 4ADP $NADPH + H^+$

$2NADH + H^+$ 4ATP

H_3C O   OH

$NADP^+$

2-Pyruvate   Acetaldehyde

$2NADH + H^+$ $NADPH + H^+$

2NAD

H_3C O   OH
OH   H H   H
H—C—C—O
H H $NADP^+$

Lactate   **Ethanol**

Aerobic/anaerobic

40

- - -> 1,2-propanediol - - - - - -> Propionate
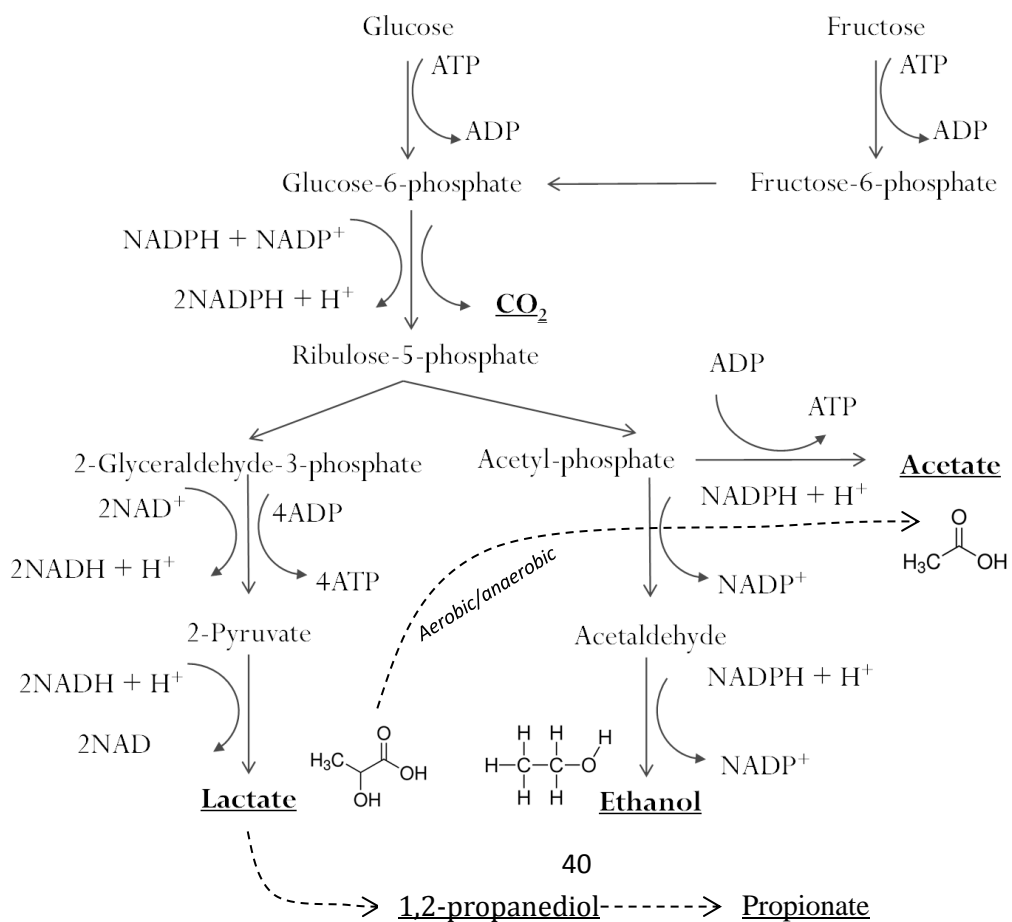
Figure 1.5. Fermentation of fructose and glucose by obligate heterofermentative LAB such as *L. buchneri, L. plantarum*, L. fermentum, Weissella cibaria* and *Leuconostoc mesenteroides* forming lactic and acetic acid in addition to ethanol and CO_2. Figure redrawn from McDonald and Henderson (1991) with putative catabolism of lactate to acetate, 1,2-propanediol and propionic acid included with dashed lines (Lindgren *et al.*, 1990, Oude Elferink *et al.*, 2001, Krooneman *et al.*, 2002). * *L. plantarum* is facultative heterofermentative.

One type of fermented feedstock with particular relevance to grain-borne DON is crimped grain (CG). The crimping of moist grain prior to ensiling is a relatively recent agricultural phenomenon championed in Finland and used extensively in northern Europe for ruminants (Olstorpe *et al.*, 2010b) with the potential for swine and poultry production (Siljander-Rasi *et al.*, 2000, Borling Welin *et al.*, 2015). It forms a valuable source of locally produced, and hence fully traceable, high quality animal feeds from a starting material that does not need to be necessarily ripe or mature. Pressed (crimped) grains are ensiled until use in airtight clamps following inoculation with LAB, buffered organic acids or chemical preservatives. Commercially available LAB inoculants such as *Lactobacillus buchneri* and *L. fermentum* have been shown to give consistently high product stability comparable with chemical preservatives (Adesogan *et al.*, 2003). However, variations in initial grain moisture, crimping, consolidation and preservation effectiveness can on occasions yield the localised growth of potentially toxigenic moulds, hence sourcing LAB inoculants able to inhibit mould germination and growth under such conditions would be well received (Borling Welin *et al.*, 2015).

The lack of the energetically costly drying step found in conventionally harvested grain and the potential for in-house production, especially during short or disrupted growing seasons, means CG will likely play a significant role sustainable feed production in the future (Jokiniemi *et al.*, 2014). The possibility that grain affected by prevailing climatic conditions may not reach peak maturity within a growing season and would then be

channelled into the crimping process could mean that such material is more likely to be contaminated with MT, for there is an observed strong correlation between wet summers and high DON levels (Langseth and Elen, 1997), and deleterious levels of DON in ensiled wheat and maize has been reported in the Netherlands (Driehuis *et al.*, 2008). Likewise, harvesting earlier could reduce the amount of accumulated DON in the final feed and the moisture content would be such that potential bacterial biotransformation could continue within the silo, compared with dried grain where DON levels are essentially fixed (Figure 1.6).

It is therefore apparent that a research opportunity exists for the development of an inoculating strain of ensiling bacteria, ideally a LAB, which can fulfil the vital role of DON biodegradation within the silo environment and storage timeframe (this research avenue is explored in chapters 3-5). Following development, the bacteria could be inoculated in a precautionary or intervention strategy in the production of CG feeds from DON-susceptible crops. Such a candidate bacteria would need to have proven biotransformation ability with the metabolites formed being of much lower toxicity. It would also be beneficial that the presence of such a strain would also inhibit the growth and virulence of undesirable fungi and bacteria, remain viable across different aerobic conditions and not significantly affect the palatability or nutritive value of the feed. There is a distinct possibility that a bacteria species exists which can be used to remove DON, yet does not share the properties associated with desired LAB metabolism, in which case a degree of co-inoculation with a proven inoculant may be prescribed such as the functional consortium approach to CG fermentation explored by Borling *et al.* (2015). Another type of fermented feed is liquid feed (LF) and is commonly used in swine
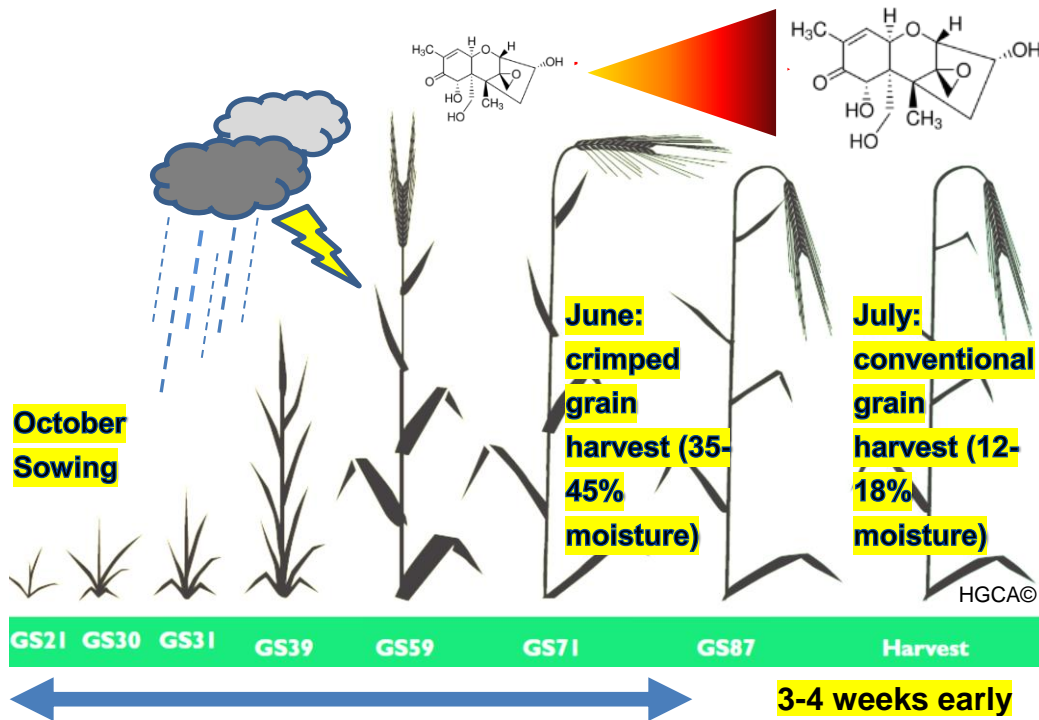
The main advantages of fermented crimped barley grain as a feedstock:

- Grain is most nutritious at 35-45% moisture (growth stage 75) and ensiled crimped feed is palatable, shown to increase productivity and low in dust.
- No energy input required for drying grain in damp harvest years.
- Harvesting earlier enables the next crop to be sown and get established 3-4 weeks earlier, especially important in northern Europe where growing seasons are shorter.
- Climatic factors may encourage *Fusarium* spp. growth/colonisation leading to ever-increasing DON levels (kg^{-1} dry weight), which could be reduced in severity by harvesting 3-4 weeks earlier than conventional grain (may be *ad hoc* intervention following risk assessment of crop for that year or FHB prevalence)
- Ensiling provides the potential opportunity to reduce the levels of DON through interaction with bacteria within the silo.

Figure 1. 6 Diagram illustrating crimped grain feedstock as a strategy of reducing DON contamination.

The original growth-stage diagram was modified from "the barley growth guide", HGCA, winter 05/06.

production. Grain-based Ingredients, including food industry co-products are mixed and spiked with LAB inoculants in common with CG, to help preserve the nutritive quality whilst reducing undesirable bacterial growth, e.g. *Salmonella* sp. (Brooks, 2008, Beal *et al.*, 2002). Additionally, such a controlled fermentation has been suggested to reduce anti-nutritional factors, including MTs such as AFB_1 (Shetty and Jespersen, 2006). Some batch ingredients are naturally more contaminated with DON than others, especially where poor quality grain is channelled into lower category use (Whitlow, 2007). Co-product from the bioethanol industry is currently increasing in volume at such a rate that incorporation into animal feeds is inevitable and is already driving LF research (European-Commission, 2006b, Gallagher, 2008, Lyberg *et al.*, 2008). The major issue with incorporating such material is that MTs are conceivably up to 3 times more concentrated than the starting material due to the process of ethanol/glycerol removal and the husk being the location of initial mould activity (Wu and Munkvold, 2008).

2 Isolation of lactic acid bacteria from wild birds for use as fermented animal feed inoculants with potential DON-detoxification capability

2.1 Aims and objectives

To isolate a range of LAB from wild birds and evaluate their DON-bioremediation properties and potential as fermented feed inoculants in terms of preventing the growth of spoilage mould.

2.2 Introduction

Fermented animal feeds have been an integral part of livestock production systems from ancient Greece through to the present day. It is however only in the past 60 years that this agricultural area, which includes: crimped grain (CG), whole crop silage (WCS), total mixed ration (TMR) and liquid feeds (LF), has been focused on for scientific evaluation and improvement; resulting in a consistently high quality and safe method of forage storage and nutrition delivery (McDonald *et al.*, 1991).

One aspect of primary concern is the effectiveness of the lactic acid bacteria to produce carboxylic acids and impart a chemical environment where the growth of spoilage organisms is inhibited (*i.e.* MT-producing moulds (Cheli *et al.*, 2013a)), the nutritional value of the forage is retained and the palatability of the feed is favourable (Dunière *et al.*, 2013). A great number of studies have focussed on the isolation and screening of LAB for this purpose in different cereal-based and whole-crop silage (Doi *et al.*, 2013, Santos *et al.*, 2013, Pang *et al.*, 2011), yet none have managed to find a LAB capable of fulfilling the task of yielding effective fermentation characteristics in addition to DON biodegradation

ability, and naturally fermented feed does not usually result in a drop in DON levels from the starting material (Garon *et al.*, 2006).

The source of bacterial inoculum for fermented whole crop/grain feeds is often the phyllosphere of the forage used (thus producing a naturally fermented product), although freeze dried commercially available LAB applied at optimal rates during processing increase the likelihood of the fermentation being undertaken swiftly by the desired LAB assemblage with the finished feed possessing high aerobic stability and consistency (Adesogan *et al.*, 2003, Filya *et al.*, 2006). These commercial strains were originally isolated from a range of environments and screened for efficient feed fermentative properties. Some strains were actually isolated from production animals such as swine in order to impart beneficial probiotic effects during GI transit (Missotten *et al.*, 2009).

The current work aims to investigate the feed fermenting potential of LAB isolated from wild granivorous (*viz.* grain-eating) birds whilst also exploring the hypothesis that a microbial detoxification element exists in granivorous birds which aids in the detoxification of toxins such as DON, and could be employed for that purpose in fermented feeds. Wild birds which habitually feed on cereal grains have two regions of the fore-gut dedicated to food processing where LAB are likely to be found. The pre-gastric crop-sac consists of an expansion of the oesophagus where food is stored for extended periods prior to peristaltically passing it to the gizzard via the proventriculus where mucus, HCl and pepsinogen are secreted. The gizzard then homogenises the food prior to further digestion in the small and large intestines. The crop-sac of birds is known to harbour appreciative counts of LAB (Baele *et al.*, 2001), especially in the wood pigeon (*Columba palumbus*) which is noted for producing "pigeon milk", a protein, cytokine and

probiotic-laden sustenance secreted for newly hatched progeny (Gillespie *et al.*, 2012). This LAB-rich environment is also where the birds will be first exposed to surface bound MT such as DON (a large proportion of which is found on the grain surface) (Kushiro, 2008). It is envisaged that the crop-sac is therefore the first organ to be exposed to MT, and it is possible that a commensal LAB assemblage is present which may possess the ability to detoxify DON therefore inadvertently counteracting DON exposure in the bird. Bacteria have already been found in the lower intestine of chickens which can detoxify DON by enzymatically biotransforming it to DOM-1 (Young et al 2007). This illustrates one detoxification mechanism that could be developed by the agri-biotechnology industry as a feed inoculant, although the strict anaerobic conditions required precludes them from use as conventional silage inoculants, whereas LAB isolated from the pre-gastric region of the gut may be more amenable to growing in fermented feeds as well as *in vivo* where they may act as probiotics. Desirable isolate properties in order of importance include: the ability to irreversibly biotransform DON into lesser toxic elements, the ability to irreversibly bind DON allowing it to pass through the digestive system intact and being an instrumental factor in producing metabolites which create an environment where DON is more prone to degradation and less likely to be absorbed *in vivo*. Further desired traits in addition to these are: the LAB is amenable to laboratory culture with swift growth in fermented feeds at a range of temperatures and matrix pH, with effective production of anti-mould metabolites such as carboxylic acids; in addition to being non-pathogenic and generally recognised as safe (GRAS, (Schultz, 1997)) with the potential for probiotic status.

An initial screening for limonene-1,2-epoxide (LIMOX) metabolising activity is proposed to be a way of finding potential epoxide moiety degradation strains of LAB. The low cost of

the material compared with purified DON and the low toxicity (used as a human food flavouring) enables a sole carbon source screening experiment to take place where LAB able to metabolise LIMOX are present as growing colonies that can be tested for DON degradation in further experiments. This is not the first study to utilise the structural homologue approach with the biodegradation of MT the goal. Theison and Berger (2005) explored the potential for finding microorganisms capable of degrading DON by screening for epoxide-hydrolase activity using epoxide-based sole carbon sources, and studies employing coumarin as a surrogate to aflatoxin B_1 (AFB_1) have proved fruitful in the sole carbon source screening for AFB_1-degrading bacteria (Guan *et al.*, 2008). The epoxide residue in DON is intrinsically linked to its functional toxicity and the removal of which to form de-epoxide-DON (DOM-1), dramatically reduces its toxicity in swine kidney cells (Kollarczik *et al.*, 1994).

2.3 Materials and methods

The steps involved in the screening process for DON degradation by LAB are shown in Figure 2.1.

| Lactic acid bacteria isolated from birds using MRS agar | Various silage inoculants and probiotic strains held by our research group |

Ability to form colonies on LIMOX agar/broth

Characterisation of strain according to growth curve properties in MRS broth

Characterisation of strain according to glucose utilisation and metabolites produced evaluated using HPLC

Determination of anti-fungal properties of bacterial medium supernatant using the mould spore inhibition assay and mycelial growth inhibition assay

Determination of deoxynivalenol metabolism in liquid medium using ELISA and HPLC

Figure 2.1. Schematic plan of LAB screening experiments.

2.3.1 Isolation of lactic acid bacteria

The wild birds used in this study were *Columba palumbus* (Wood Pigeon), *Alectoris rufa* (Red Legged Partridge), *Phasianus colchicus* (Pheasant) and *Prunella modularis* (Dunnock) and were processed in November 2008. The Dunnock was found freshly dead due to natural causes (flying into a window) and the game birds were purchased from a local butcher as duplicates, sourcing locally in Devon, UK (long legged in the feather, *i.e.* not gutted). Birds were stored at 4 °C during the period between death and processing. Dissection was undertaken with sterile equipment that included clamps enabling the sectioning of the gut into crop and gizzard regions to prevent cross contamination. An

incision was made in the side of each organ region and the whole contents were squeezed into a petridish to record the contents description and estimated proportions, then mixed into a pre-weighed bottle of 100 ml autoclaved PBS and homogenised using a Waring (USA) laboratory blender. This was serially diluted 10-fold in PBS and enumerated using MRS agar (de man, Rogosa, Sharpe medium, pH 5.4; Oxoid, Basingstoke, England) for LAB (37 °C, 5% CO_2, 48 h). The total counts were additionally counted using fluorescent microscopy with DAPI stained material on a haemocytometer. A representative selection of colonies were selected from each bird species (crop-sac and gizzard) according to morphological characteristics (size, colour, colony shape and texture and incubated to stationary phase in MRS broth (50 ml centrifuge tubes incubated statically with zero head space, 37 °C, 5% CO_2, 48 h) prior to harvesting by centrifugation (2000g, 20 min) and storage in cryopreservation medium (sterile 30% glycerol in MRS broth) at -80 °C (Table 2.1).

Table 2.1. Isolates and commercially available additives used in the current study.

Strain	Origin/application	Reference/Source
L. fermentum (**NCIMB 6991**)	Silage additive	UoP collection, Oregon State Collection
L. buchneri (**DSM 40788**)	Silage additive	Biotal, UK
P. acidilactii (**PD-962**)	Silage additive	Medipharm, Chr. Hansen, Denmark
L. plantarum (**DSM 16568**)	Silage additive	Medipharm, Chr. Hansen, Denmark
P. acidilactii (**CNCM MA 18/5M**)	Probiotic feed additive	Lallemand, France
5a (*L. plantarum*)	Chicken isolate	Josef Nissimov, Israel
5b (*Leucobacter albus*)	Chicken isolate	Josef Nissimov, Israel
L. sakei	Small intestine scrape, chicken	Savvidou (2009)
#9 (*L. plantarum*)	Small intestine scrape, chicken	Savvidou (2009)
Phy2c1	Pheasant crop	This study
Phy2c2	Pheasant crop	This study
PayC6	Partridge crop	This study
Payg5	Partridge gizzard	This study
Pi6(3)	Pigeon crop	This study
PiyG7	Pigeon gizzard	This study
Dun1	Dunnock crop	This study

2.3.2 Selection for growth using LIMOX agar

The gradient plate technique was chosen for the initial screening of all strains. LIMOX medium was formulated as 1 L of minimal salts medium (MSM) agar (12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3.1 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 10 g agar and 1 ml sterile $MgSO_4$ added post autoclaving) with 1% 0.2 μm filter sterilised Limonene-1,2-epoxide (v/v) and 0.05% Tween-80 added at 47 °C before pouring into glass plates (to prevent chemical interaction of polystyrene with LIMOX). 10 ml of LIMOX medium was added to plates positioned on a bench sloped with an angle of 25° and left to set before adding 10 ml of MSM agar (no carbon source) on a flat bench to give a double layer (Figure 2.2). Confluent culture grown in MRS (20 μl) was spread onto each plate and incubated for 7 d (37 °C, 5% CO_2). Those strains exhibiting growth by forming colonies were inoculated into fresh MRS broth and stored for the next screening stage. Positive controls for LIMOX or DON metabolism were not included as they were not commercially available. The theoretical solubility of DON and LIMOX is also shown for comparison (Figure 2.3).
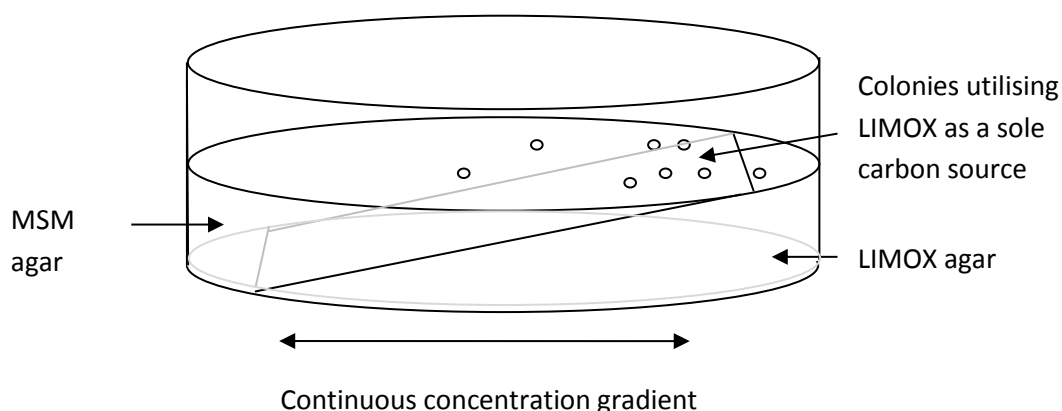
Figure 2. 2. Method of isolating LAB able to utilise LIMOX as a source of carbon. The thickness of the over-riding minimal medium agar regulates the amount of compound available by diffusion.

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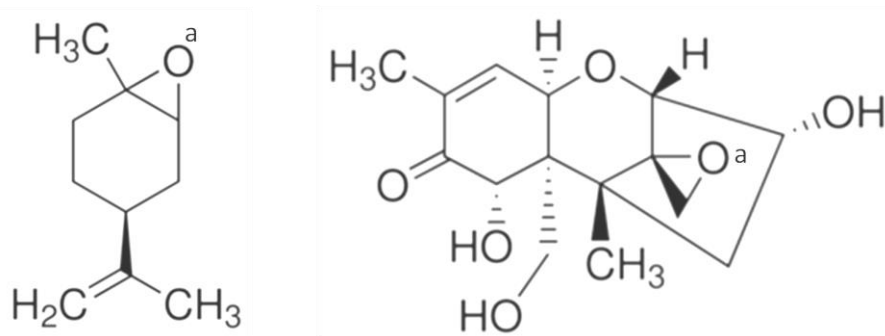
	Limonene-1,2-epoxide	Deoxynivalenol
Formula and mass	$C_{10}H_{16}O$, 152.23 Da	$C_{15}H_{20}O_6$, 296.32 Da
Water solubility at 25 °C, estimated using US Environmental Protection Agency's EPISuite™	0.14 g/L	51.75 g/L

Figure 2.3. Comparison of molecular structure and estimated water solubility between limonene-1,2-epoxide and DON showing epoxide moiety (a). Estimation Program Interface (EPI) Suite was accessed through Chemspider (www.chemspider.com**)**

2.3.3 LIMOX broth turbidimetric analysis

LIMOX broth was formulated as above, but without agar. Confluent culture (50 µl) was added to 50 ml of LIMOX broth in glass Schott bottles and incubated at 30 °C with lids sealed. Suspended culture (50 µl) was removed at 1 h intervals and analysed at 600 nm for increases in turbidity.

2.3.4 Detection of DON metabolising ability

LAB able to produce colonies on LIMOX agar were further tested with genuine DON. DON was purchased from Sigma-Aldrich as a freeze dried powder (5 mg) and re-suspended in HPLC-grade ACN to a concentration of 5 mg/ml. This was verified spectrophotoscopically by diluting 1000-fold and using the Beer/Lambert equation:

Absorbance = ε x concentration (mol) x pathlength (cm). Absorbance = 0.134, Molar extinction coefficient (ε) = 6805 L/cm/mol at 217nm (Krska et al., 2007), Pathlength of far UV grade cuvette = 1cm,   0.134/6727 x 1 = 1.97 x 10^{-5} =19.69 µM in a 1000-fold

dilution in ACN=19.69 mM in initial stock. 0.01969 M x 296.32 = 5.83 mg/ml (16.70% more than stated on label).

This 5.83 mg/ml stock was spiked into 20 ml of MRS broth at a concentration of 5000 µg/L (16.8 µl, 5000 ppb) with the same volume of ACN added to negative control MRS. These treatments were put on 48 well plates (0.5 ml) and inoculated with 10 µl of each specific strain in 24 h growth phase. The plates were incubated in a plate reader (Infinity-200, Tecan, Austria) to enable differences in growth rates to be monitored. The entire contents of each well were then collected at 48 h in pre-weighed 0.5 ml tubes prior to centrifugation at 10000g. Supernatant (400 µl) was removed and stored leaving the pellet fraction, which was weighed to determine the proportion of DON in each fraction, hence resolving the degree of DON binding in the pellet if present. The experiment was undertaken in triplicate and the determination of DON undertaken using a RIdascreen DON™ competitive ELISA (Rhône-Biopharm, Glasgow, UK) following the procedure outlined in the kit (Figure 2.4). A minimum R^2 value of 0.99 was required for the standard curve. Following the spiking of MRS with DON it was very difficult to use the cell-free supernatant (CFS) directly with the ELISA. A very high false positive was found in this competitive ELISA indicating that a cross-contaminating or denaturing agent was present. Presumably, this agent will have a similar effect on the immunoaffinity columns needed for HPLC verification of DON and D3G. To remedy these effects a greater DON concentration was used in the LAB incubation (5000 µg/L instead of 50 µg/L) to allow for a 100 fold dilution prior to adding to the ELISA. This prevented any contaminating effects attributed to the acidity, ionic strength, detergent or protein content of the MRS broth. In addition, the effect on DON recovery by the low inherent pH of CFS was confirmed by

using a neutralising step with differing amounts of 1N NaOH and measuring the ELISA

recovery before and after as well as the pH (micro-pH electrode, Fisher).
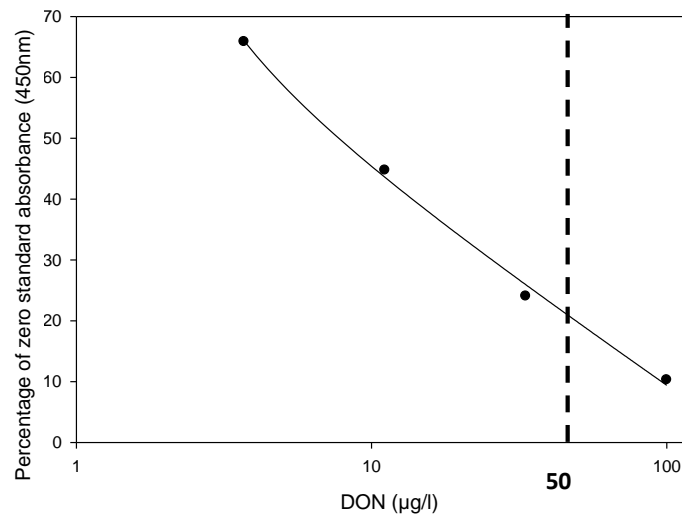
Figure 2.4. DON Standard curve for competitive ELISA. Standards used were 0, 3.7, 11.1, 33.3, 100 µg DON/l in water. Absorbance values are given as a percentage of 0 µg/L DON absorbance. The non-linear regression fit is Y = 76.288+14.581(lnX - 1.673) with an R2 of 99.6%. The dotted line indicates where the control DON level should appear following a 100x dilution of the 5000 µg/L treatment medium.

2.3.5 Evaluation of LAB growth and metabolism characteristics

On the basis of population growth curve properties and evaluation of simple sugar

metabolism allows the rapid determination of co-occurring strains and enables the

selection of bacteria likely to be of use in fermented liquid animal feeds. Overnight

cultures (37 °C, 5% CO_2, 48 h) were diluted to a uniform turbidity of 0.2 in micro-cuvettes

(600 nm) and 10 µl was added in replicate to 240 µl MRS broth in 96 well plates, flushed

with 5% CO_2 and read at 600 nm every 30 min for 48 h (Infinity-200, Tecan, Austria). The

data was analysed using the Magellan 6.5 software for the following parameters; curve

slope, OD peak. After 48 h, the incubated MRS medium was centrifuged (10000g, 20 min)

and filtered to 0.22 µm before storage at -80 °C. HPLC analysis was performed using a

Phenomenex organic acids column (7 mm x 300 mm) with nitrogen degassed 2.5 mM

H_2SO_4 as the mobile phase in isocratic mode (0.5 ml/min). The sample (500 µl) was

spiked with 20 µl of 7% H_2SO_4 (v/v) to protonate the acids and stabilise the column and 10

µl of sample was injected with the aid of a Gynotek autosampler and run for 30.5 min.

Peak analysis was undertaken using Chromeleon-6™ software (Figure 2.5).
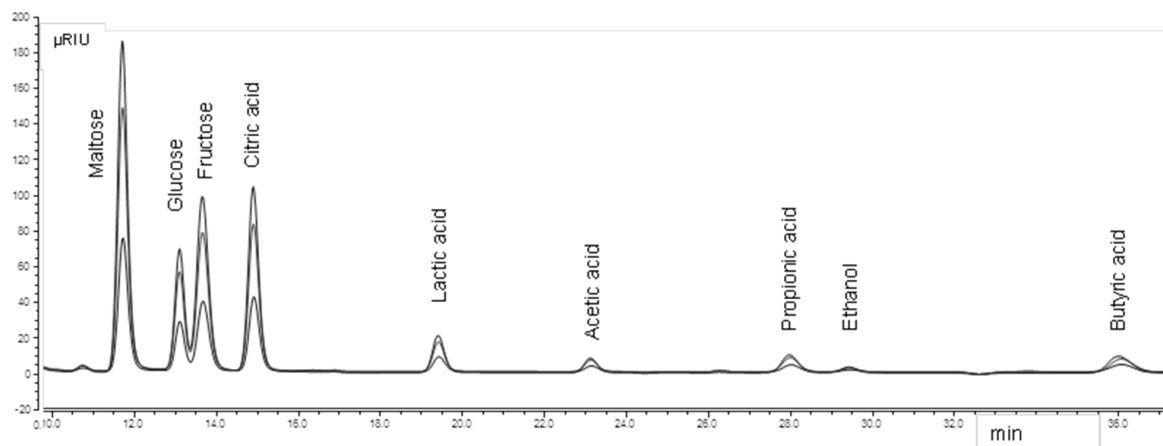
Figure 2.5. A representative HPLC chromatogram of mixed monosaccharide and metabolite standards at 25, 50 and 100 µM measured using refractive index detection.

For the organic acid samples, it was necessary to both dilute the sample down and

remove proteins that produced an irresolvable hump preventing the identification of acid

peaks. Samples were diluted 1:10 with distilled water prior to protein precipitation.

Addition of perchloric acid is a standard protein precipitation technique. The sample to

be treated was added to an equal volume of 0.5 M perchlorate and then neutralised with

twice the sample volume of dipotassium carbonate (1 M) and centrifuged at 10000*g* to

leave a purified supernatant (noting the additional dilutions).

$$2HClO_4 + K_2CO_3 \rightarrow 2KClO_4 + CO_2 + H_2O$$

2.3.6 Determination of mould conidia spore inhibition by bacterial metabolites

Inhibition of growth was determined using the turbidimetric approach in common with

the bacterial growth assays with a method adapted from Lavermicocca *et al.* (2003).

Aspergillus niger was employed as a surrogate toxigenic spoilage mould for its ease of

laboratory culture and sporulation (CABI 393094). Fusarium was not used for this initial study due to inconsistent sporulation and this assay was developed for spoilage mould rather than phytopathogenic species. *A. niger* was grown on potato dextrose (PD) agar (Oxoid) following spore inoculation. The generation of spores is apparent as black structures in localised areas on the mycelia. These are washed off using 7% NaCl/0.05% TWEEN-80 to facilitate spore removal and separation, and then counted with a haemocytometer before diluting to 10^4 spores/ml in distilled water. The spore solution for each experiment was enumerated on PD agar and viable counts of 4000 CFU $\pm$ 1000/ml were found across all experiments.

Serial dilutions of 0.2 µm filter sterilised LAB supernatant (48 h culture in sealed 50 ml centrifuge tube at 37 °C and centrifuged at 10000*g*) were added to a 96 well plate (190 µl). Spore suspension (10 µl) was added to each treatment well and the plate was incubated at 30 °C for 48 h prior to an absorbance reading (600 nm) where each individual well is scanned in 16 separate positions and an average taken (mycelia growth is less homogenous than bacterial growth). Positive controls for germination inhibition were made in pure MRS with 10-fold serially diluted concentrations of acetic, propionic, butyric, lactic from 1 M in MRS and phenyllactic acid from 250 mM (>99% purity, Sigma)

Due to the spent medium being inherently low in nutrients following bacterial utilisation, glucose (20 g/L) and lab-lemco (8 g/L) was later added to the medium before adding the spores. A 1:1 mix of cell free supernatant (CFS) and MRS was used which provided the nutrients required for germination and the inhibiting compounds produced by the LAB, albeit 50% diluted. A heat inactivated CFS (1 h at 95°C in water bath) diluted by 50% with MRS enables the elucidation of a heat labile based inhibition agent.

2.3.7 Statistics

Growth curve data was first characterised in terms of; gradients, maximum OD, time to reach specific OD, using the Magellan v6.1 software integral to the plate reader. Data was then compared using Minitab (version 16) and correlations made with other endpoint parameters if available. Depending on normality and homogeneity of variance, a 1-way ANOVA with Tukey's pairwise comparison was carried out.

2.4 Results

2.4.1 Isolation of LAB from birds

The total cell counts recorded microscopically for the three main bird species (the Dunnock crop-sac was immersed in broth for 24 h prior to plating due to the initial dilution not yielding colonies and absolute counts were therefore unavailable) varied between 3.50×10^5 and 2.28×10^7 cells/g dry matter in the crop-sac of which the proportion that were able to form colonies on MRS and were therefore likely to be LAB was a large proportion when considering the pheasant and less in the other species (Table 2.2). In the gizzard total counts ranged between 1.29×10^4 and 5.91×10^5 of which few were viable when plated out illustrating the transit of LAB from the crop-sac to the lower intestine is prevented on the whole by the harsh chemical conditions in the gizzard. It could be hypothesised that the LAB able to be isolated from the gizzard are more likely to have a probiotic role to play in any fermented feed scenario. The crop-sac contents were cereal based for the Pheasants and Dunnock, grass and cereals for the Partridge and ivy berry/foliage based for the wood pigeons. This is possibly an indication of seasonal availability and human provision, yet the bacteria isolated may well be omnipresent as a commensal assemblage within the birds GI tract in all seasons.

Table 2.2. Description of wild bird crop and gizzard contents given as means of triplicate measurements. The total bacterial count was undertaken using fluorescent microscopy of diluted samples and the viable LAB plated on MRS agar with the percentage viable of the total cell count in shown in brackets.

	Crop sac	Gizzard	Crop sac	Gizzard	Crop sac	Gizzard
Male Pheasant	6.00×10^5	6.02×10^4	4.21×10^4 (6.83%)	ND	90% whole wheat, 10% grit	80% ground wheat, 20% grit
Female Pheasant	3.50×10^5	2.49×10^5	1.06×10^4 (3.03%)	ND	90% whole wheat, 10% grit	80% ground wheat, 10% grass, 10% grit
Partridge A	1.51×10^5	1.34×10^4	7.77×10^3 (5.15%)	ND	70% whole wheat, 30% grass	60% ground wheat, 30% grass, 10% grit
Partridge B	2.28×10^7	1.29×10^5	ND	ND	100% grass	90% grit, 10% grass
Wood pigeon A	2.94×10^6	3.51×10^4	2.94×10^3 (0.10%)	8.85×10^{-1} (0.25%)	100% ivy berries	80% ground ivy berries, 20% grit
Wood pigeon B	8.17×10^6	5.91×10^5	ND	1.83×10^0 (0.00%)	100% unidentified brown Leaf material	90% grit, 10% wheat
Dunnock	-	-	-	-	100% Wheat	-

2.4.2 Screening for limonene-1,2-epoxide metabolism ability.

In an initial experiment with 4 representative LAB strains inoculated into LIMOX broth it was apparent that all exhibited an initial rise in population followed by a decline after 24 h indicating that the 1% *(v/v)* LIMOX was not initially toxic enough to halt growth but was equally not able to support further proliferation as a source of carbon, as the curves were similar to that found in M9 medium where no carbon source was present (Figure 2.6). A second experiment using glass Petri dishes to avoid the action of this volatile monoterpene oil on polystyrene (as indicated in a pilot experiment) and increasing the concentration of limonene-1,2,-epoxide to 2% with and without Tween-80 to aid substrate solubility for bacterial utilisation over 7 d (37 °C, 5% CO_2) yielded a better response than the previous experiment.   Colonies formed around the droplets of substrate held within the agar matrix confirming utilisation of hydrophobic compounds is possible by this mechanism. The viability of colonies was confirmed microscopically and by transferring a portion of underlying agar to a tube of MRS broth. The fact that several strains are able to exhibit a form of growth on LIMOX agar is not proof that any of these positive strains interact with the epoxide moiety in order to derive energy from the LIMOX compound (Table 2.3). It is conceivable that an oxidation is occurring to form a more soluble entity that can be utilised more readily and does not share any of the desired DON-like structural properties. Some strains not showing LIMOX activity, but having favourable growth characteristics were used in the DON metabolism experiment for discovering whether such a sole carbon source LIMOX technique is worthwhile. Differences in growth between liquid and solid LIMOX medium may be explained by differences in oxygen levels, although this was not explored further. Use of coumarin and

umbelliferone as surrogate AFB$_1$ homologues proved to be difficult due to their very low solubility, and were abandoned (data not shown).
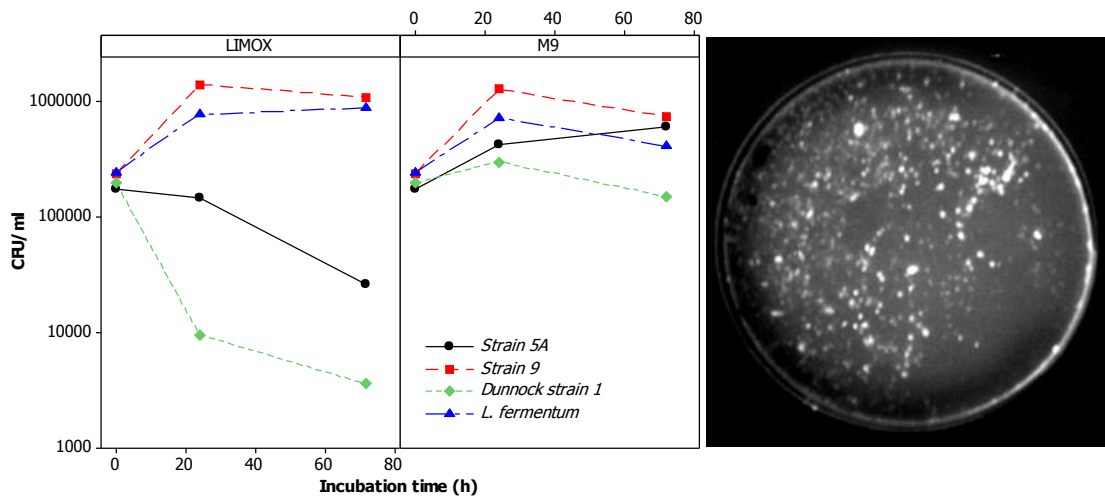
Figure 2.6. Effects on bacterial growth of using Limonene- 1,2,-epoxide (LIMOX) as a sole carbon source in minimal medium broth (MSM, pH 6.2, 30 °C). The population was determined by serially diluted plate counts on MRS agar (CFU). The four representative strains used in this enrichment culture were randomly chosen for this pilot experiment. Right; colony formation on LIMOX agar by *L. planta*rum (in glass Petri dish, image taken using fluorescent illumination.)

Table 2.3. Summary of LAB colony formation on LIMOX agar over 7 d. Plates given a visual score of response in comparison with un-inoculated equivalent plates (++ = presence of several large and small colonies, + = sparse growth, absent growth left blank). (a) signifies that LAB was isolated from this plate for the DON experiment whereas (b) signifies that the isolate was taken from the original stock culture.

Strain	LIMOX	LIMOX + TWEEN-80
L. fermentum (UoP)	+	+ [a]
L. buchneri (Biotal)	+	+ [a]
P. acidilactii (Medipharm)	+	+ [a]
L. plantarum (Medipharm)	++	++ [a]
P. acidilactii (Lallemand)	+	+ [a]
5a (Chicken)	+	+ [a]
5b (Chicken) [b]		
L. sake (Chicken)	+	+ [a]
#9 (Chicken) [b]		
Phy2c1 (Pheasant crop)		+ [a]
Phy2c2 (Pheasant crop)	+	
PayC6(1) (Partridge crop)	+	
Payg5 (Partridge gizzard)	+	+ [a]
Pi6(3) (Pigeon crop) [b]		
PiyG7 (Pigeon gizzard) [b]		
Dun1 (Dunnock crop)	+	+ [a]

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2.4.3 Determination of DON removal by lactic acid bacteria *in vitro*
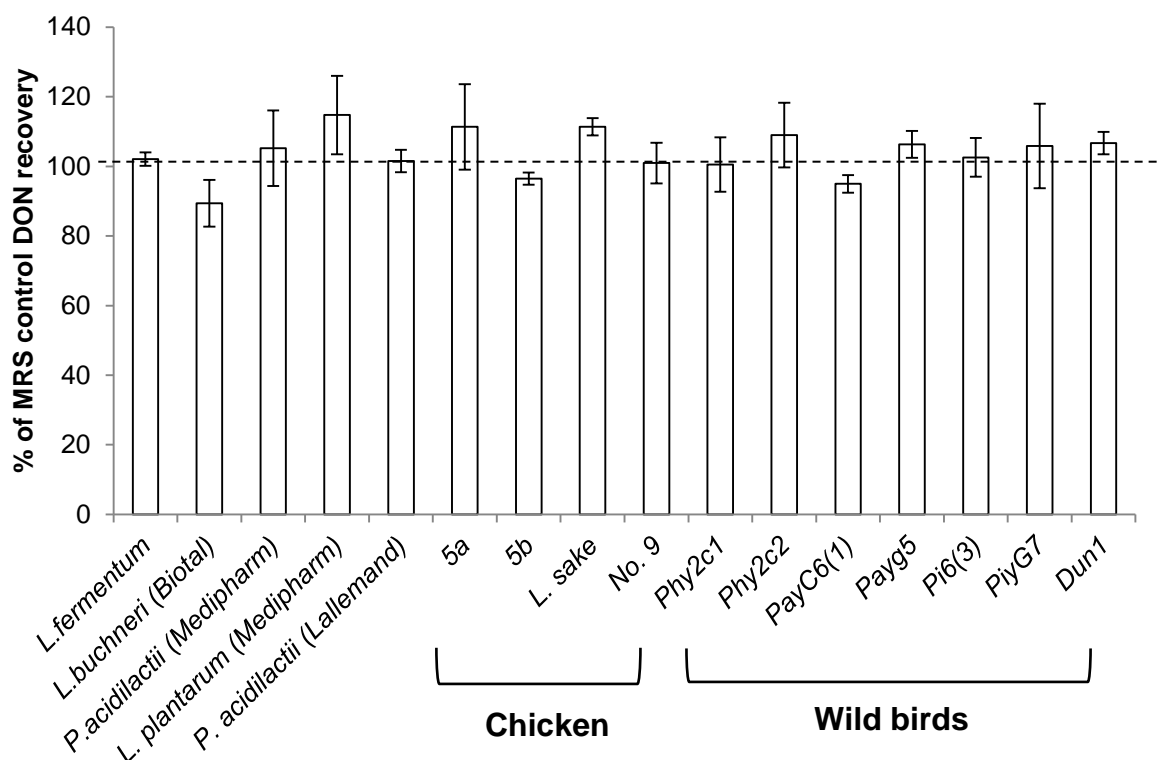
Figure 2.7. Percentage DON recovery following incubation with different LAB for 48 h in MRS broth at 37 °C. Mean of triplicate experiments of duplicated ELISA wells shown with SE.

All the strains were found to have insignificant DON biotransformation and binding activity, with mean recoveries similar to that of the starting level (Figure 2.7). There were no significant differences in activity between the strains (ANOVA: F= 0.81, P= 0.662, n = 16). As these measurements were made using the supernatant of each exposure and the DON concentration had not changed, it can be assumed that the proportion of DON in the bacterial pellet is equal in terms of medium volume and no binding of DON has occurred. Furthermore, the presence of DON in the medium at the concentration used did not influence the growth parameters of the LAB (data not shown). It is worth remembering, that the ELISA kit used is subject to cross-contamination by differing DON derivatives such as 3-Acetyl-DON and 15-Acetyl DON, so it is important to double check the suite of

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metabolites produced using HPLC, although the almost 100% recovery in the current experiment suggests DON is not being metabolised to a derivative dissimilar to DON.
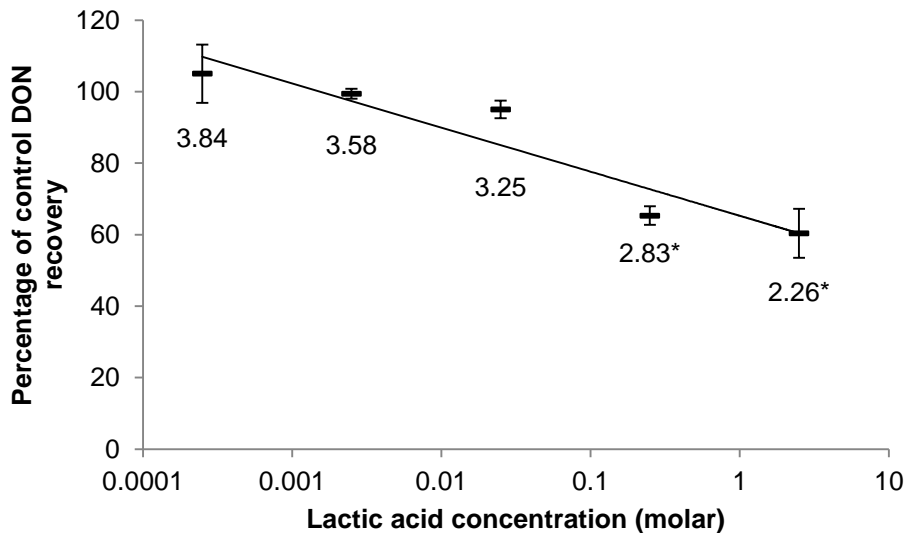
Figure 2.8. The effect of lactic acid treatment on DON recovery (48 h at 37 °C). The mean percentage of control DON recovery (pH 6.2) measured by ELISA is shown with SE of 8 replicates per treatment. The regression fit: % reduction in DON = -5.35ln[lactic acid] + 65.27, accounts for 89.3% of the variance of the data. The pH of each treatment is given beneath each data point with the asterisks denoting a significant (*P*<0.05) reduction in DON recovery compared to the control.

A one-way ANOVA indicated lactic acid reduced the recovery of DON significantly (F = 42.2, *p*<0.001, n = 48), although without confirmation of the mechanism by HPLC analysis and identification of degradation products, it is not clear if the structure of DON was irreversibly modified. The use of a positive control DON-metabolism strain would have greatly benefitted this part of the study, although presently the only commercially available bacterium able to perform this task does so under strict anaerobic conditions *in vivo* (Binder *et al.*, 1997).

Any influence the pH of the sample may have on the functioning of the ELISA was evaluated by utilisation of a pH increasing step, yielding a spread of pH values that showed no significant increase on recovery (Figure 2.7, *p* =0.619, n = 24 (12 treatments in

duplicate), suggesting elevated concentrations of lactic acid does effect the levels of measurable DON using ELISA.
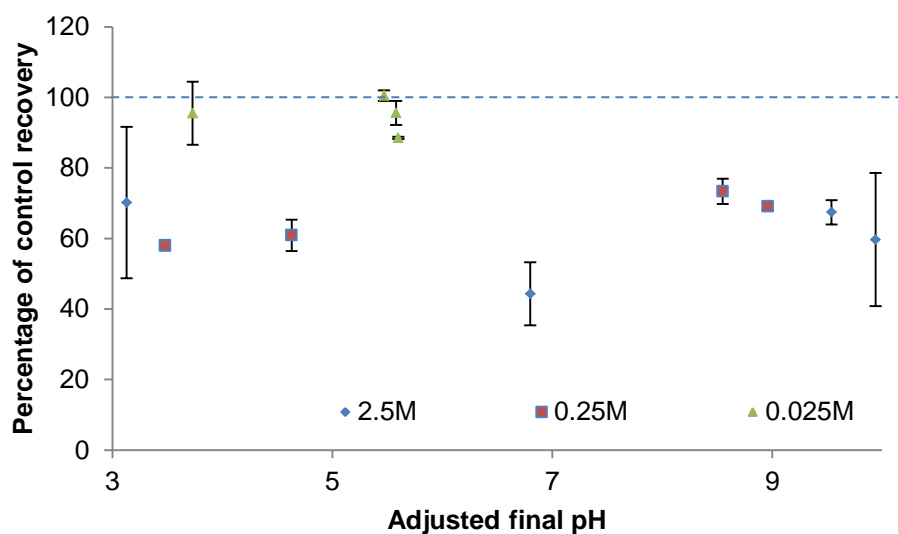
Figure 2.9. Percentage of control (pH 6.2) recovery of DON using ELISA following treatment with lactic acid and then adjusting the pH to different levels using NaOH.

2.4.4 Evaluation of mould inhibition potential and carboxylic acid production

The validation results of the *Aspergillus niger* conidia germination assay illustrated that it is a sensitive assay for determining conidia germination and growth inhibition by different compounds and growth was not limited by the use of MRS broth. Propionic acid was the most potent inhibitor of germination with EC_{50} values 100 fold lower than lactic acid (Figure 2.10, Table 2.4).   Much higher concentrations were required for complete inhibition however, perhaps a measure of the tenacity of the mould which was able to grow albeit at a reduced rate. The influence of pH was found at higher concentrations, although the presence of Cl^- ions could be the dominant factor rather than H^+ in the HCl treatment.
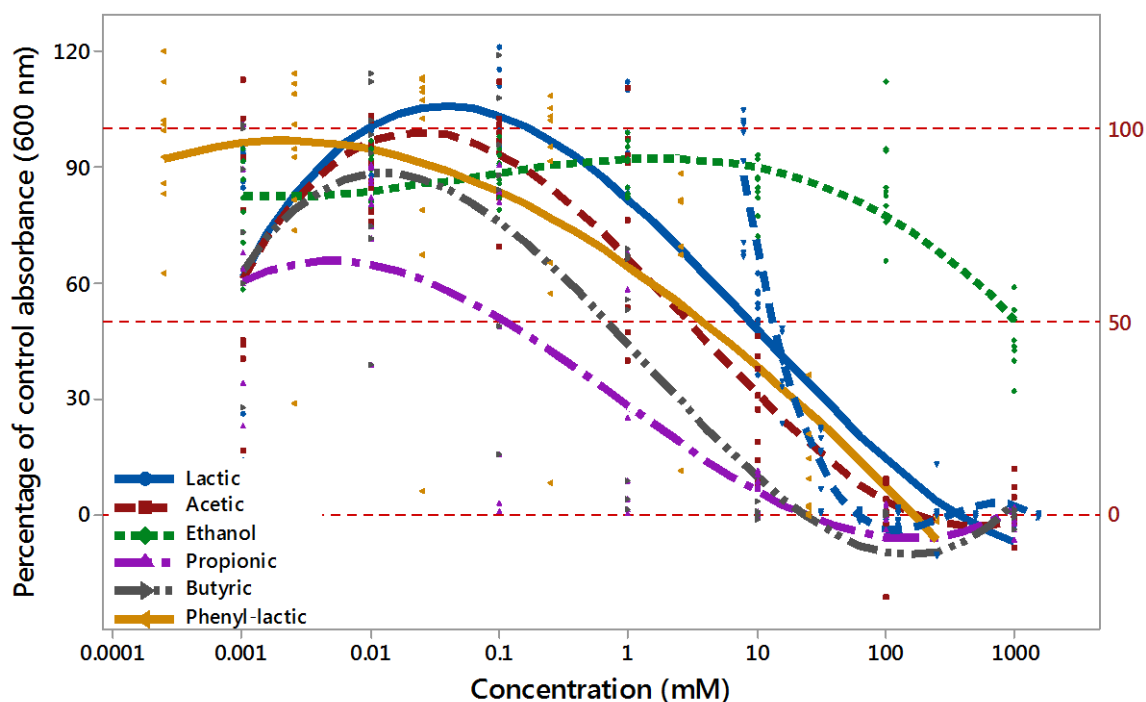
Figure 2.10. The effect of different carboxylic acids and ethanol on the germination and growth of *Aspergillus niger* conidia in MRS. Mean of triplicate experiments shown without error bars for clarity.

Table 2.4. The concentration of carboxylic acids and ethanol required to reduce mould germination and growth over 48 h (37 °C) by 50% compared with the MRS broth control.

Treatment	pKa	EC_{50} concentration mM (mg/ml)	Non- linear cubic regression fit	R^2 (%)
Lactic acid	3.86	9.05 (0.82)	$81.76 - 29.71x - 6.054x^2 + 2.028x^3$	83.2
Acetic acid	4.75	3.91 (0.23)	$66.46 - 33.51x - 4.067x^2 + 2.578x^3$	80.3
Propionic acid	4.87	0.08 (0.01)	$28.21 - 23.86x + 0.3130x^2 + 1.560x^3$	59.7
Butyric acid	4.82	0.50 (0.04)	$44.06 - 36.05x - 1.209x^2 + 2.891x^3$	69.0
Phenyl-lactic acid	3.72	2.30 (0.38)	$64.30 - 22.77x - 3.344x^2 + 0.2353x^3$	68.9
Ethanol	16.00	~1000 (~46.07)	$91.99 + 1.428x - 2.837x^2 - 0.7603x^3$	65.7
Hydrochloric acid	<1.00	15.52 (0.57)	$385.9 - 495.1x + 205.8x^2 - 27.78x^3$	65.7

The assay was found to be well suited to evaluating the inhibitory potential of LAB metabolites in CFS. Strains producing enough carboxylic acids to reduce the pH to below 4.4 were able to prevent germination outright in 1:1 diluted medium, with pH indicative of total carboxylic acid content largely responsible (Pearson correlation coefficient = -0.890, *P*<0.001 for pH, -0.792, *P*<0.001 for total acids). It is likely that strains not producing sufficient lactic acid for inhibition are also slow to exhaust nutrients as

indicated by the differential mean growth rates and the resulting CFS may be more favourable for conidia growth as is found with Phy2C1(2) and PiyG7 (Table 2.5). Supplementation of CFS with glucose and Lab-lemco (meat extract) did little to improve conidia growth in the inhibiting CFS treatments, yet diluting CFS 1:1 with MRS helped elucidate the strains with lower inhibition potential. The most inhibiting strains were characterised as having a high mean growth rate (Pearson 0.558, P = 0.025), high maximum OD at 48 h (Pearson 0.504, 0.046) and high lactic acid content (Pearson 0.795, P<0.001) that was likely the main heat resistant inhibition agent with the other acids contributing to the effect (Figure 2.11). The unusual level of propionic acid production in *L. plantarum* PayG5 and DUN1 (Figure 2.12) can be explained by the anaerobic metabolism of lactic acid by these bacteria, a phenomenon recorded by Driehuis *et al.* (1999) and Lindgren *et al.* (1990) in *L. buchneri* and *L. plantarum*, where other electron acceptors such as shikimate or oxaloacetate enable lactate catabolism and reinforce the antifungal capacity of the CFS by production of acetic, 1,2-propanediol and propionic acid.
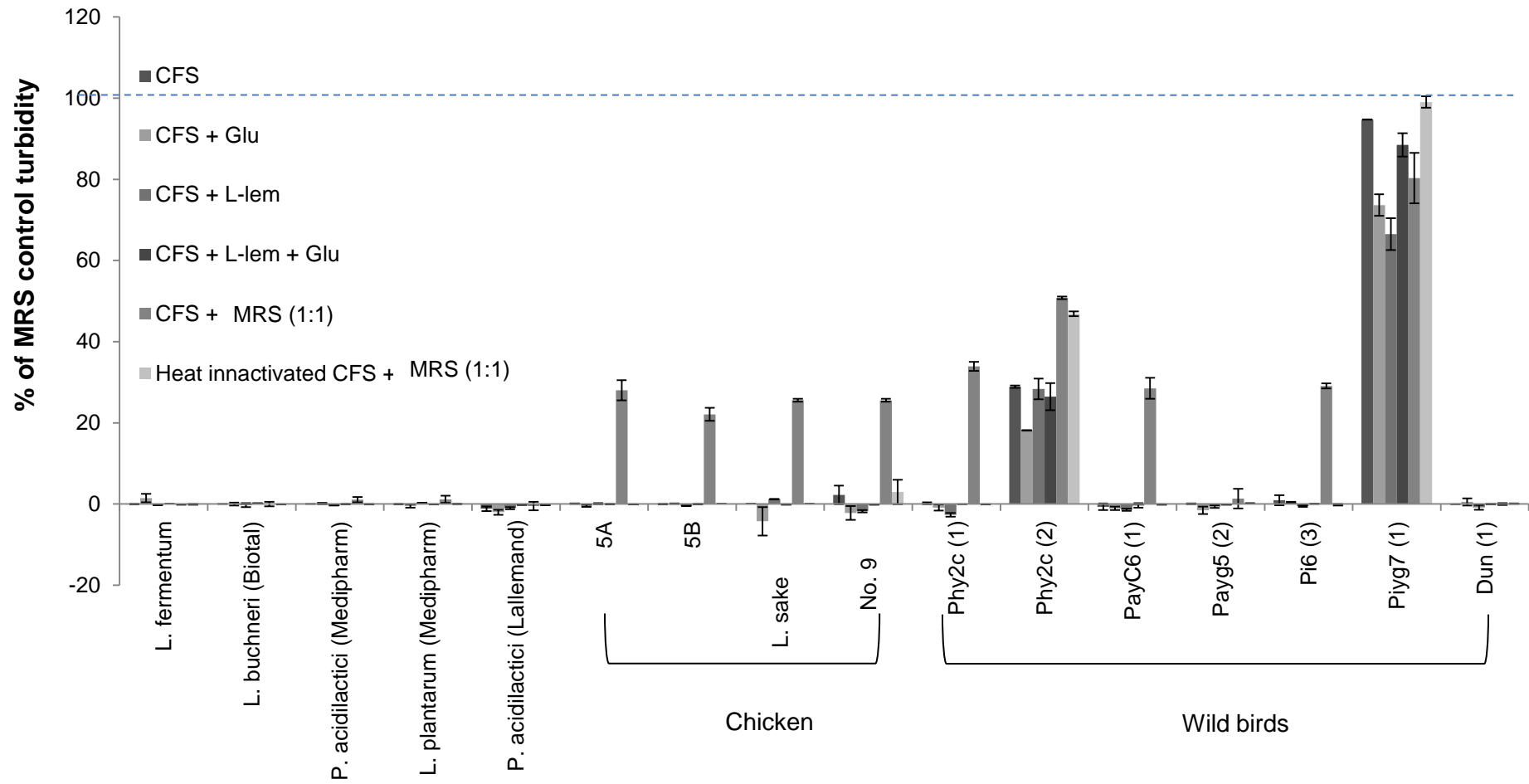
Figure 2.11. Percentage of control (MRS broth) mould conidia germination and growth in cell free supernatants (CFS) of a selection of lactic acid bacteria isolated from wild birds, broiler chickens or commercially available silage inoculants. Additional medium components and treatments enable identification of inhibitory elements in CFS. The mean of duplicate experiments (with triplicate wells) is shown with the standard error.
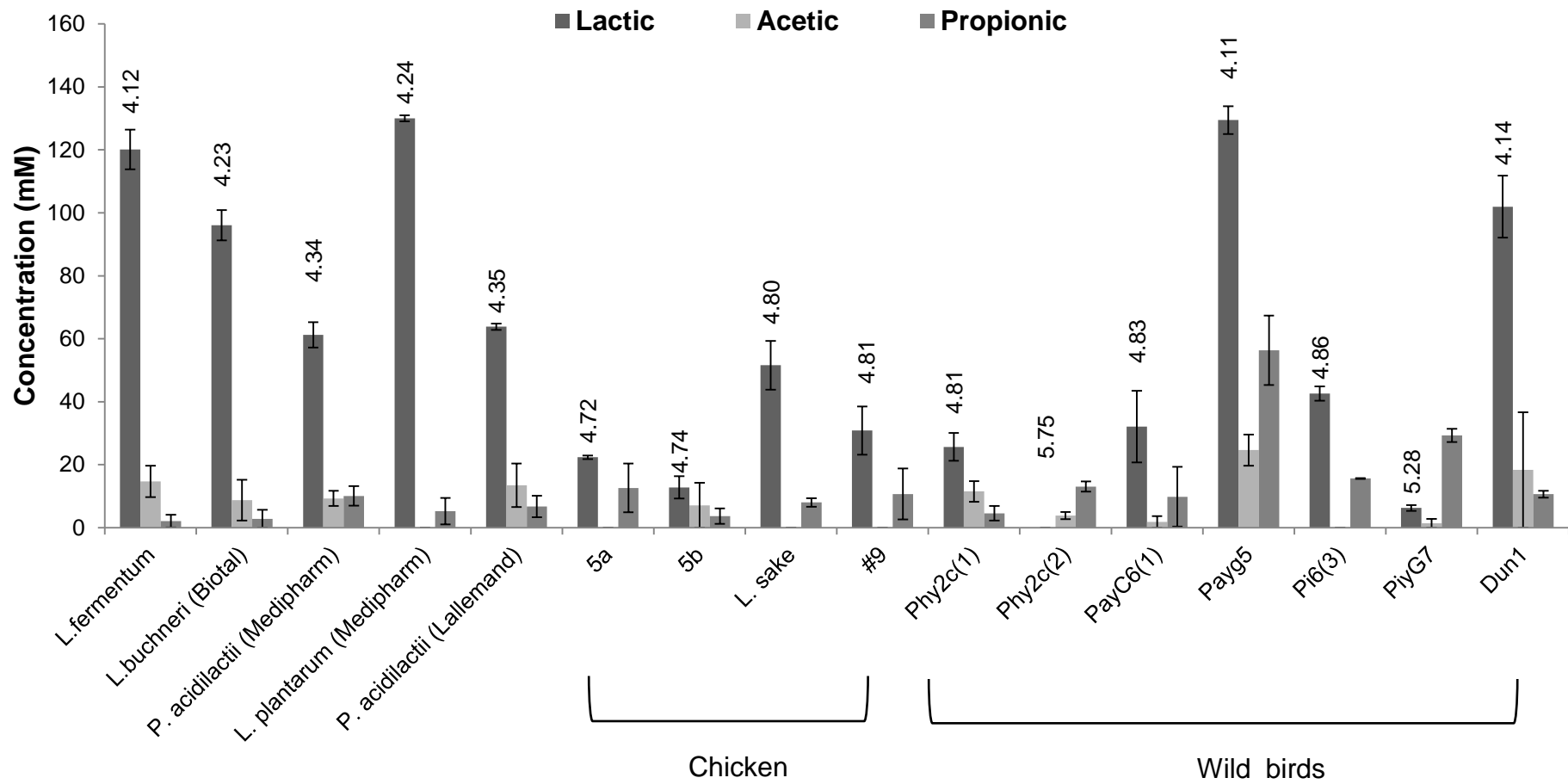
Figure 2.12. The concentration (mM) of lactic, acetic and propionic acids produced by each strain of bacteria in MRS broth incubated for 48 h at 37 °C (5% CO_2).

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Table 2.5. Growth characteristics of each strain in MRS (48 h at 37 °C, triplicate wells).

Strains	Mean Slope (OD/min x 10^{-4})	Max Slope (OD/min x 10^{-3})	Time to max slope (min)	Max OD	Time to max OD (min)
L. fermentum (UoP)	5.22	2.26	960	1.11	2010
L. buchneri (Biotal)	5.09	2.56	1340	1.02	2240
P. acidilactici (Medipharm)	4.36	3.09	940	1.04	1610
L. plantarum (Medipharm)	4.99	3.06	1160	1.04	1850
P. acidilactici (Lallemand)	4.76	2.93	1130	1.04	1750
5A	3.22	3.19	580	0.88	2800
5B	3.24	3.23	600	0.88	2770
L. sake	4.95	2.76	1210	1.02	1780
No. 9	3.92	2.91	840	0.97	1550
Phy2c (1)	3.74	2.83	830	1.02	1370
Phy2c (2)	3.24	3.23	600	0.88	2770
PayC6 (1)	4.93	2.81	1170	1.02	1750
Payg5 (2)	3.52	2.46	640	0.94	1730
Pi6 (3)	4.04	2.14	790	0.92	2050
Piyg7 (1)	1.28	7.07	860	0.33	2260
Dun (1)	3.84	2.88	810	1.03	1350
Mean	4.02	3.09	903.75	0.95	1977.50
Standard Error	0.26	0.29	62.12	0.05	122.72
Coefficient of variation (%)	0.25	0.36	0.27	0.19	0.24
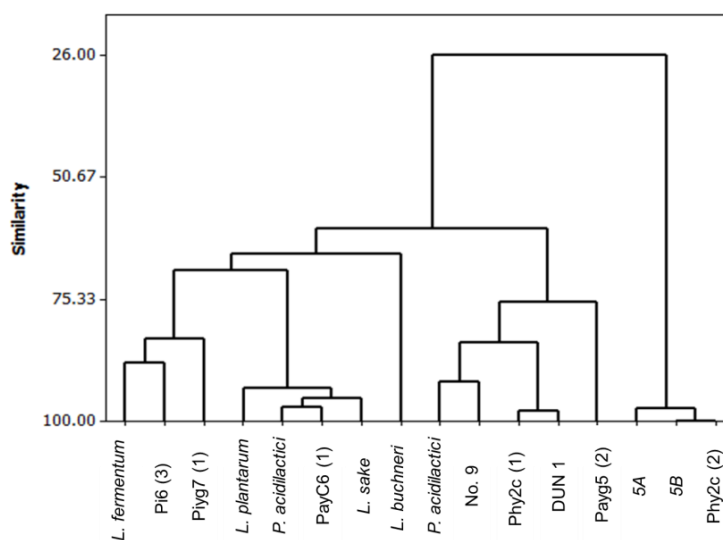
Figure 2. 13. Dendrogram of strain growth parameters in terms of similarity.

Comparing the turbidimetric growth parameters of each strain in MRS broth aids in

understanding which strains have shorter lag phases, delayed or shortened logarithmic

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phases and elevated maximal OD, which allows strains to be putatively clustered into similar trait groups (Figure 2.13).

2.5 Discussion

DON is both renowned for its potent toxicity and resistance to metabolism and degradation; hence the identification of a method of biological removal would be well received by the cereal growing and livestock rearing industries. The potential for finding a species of bacteria capable of deactivating DON *in vitro* that could be then developed to remediate contaminated cereal grains is explored in the current study using various screening techniques and a bank of LAB isolated from wild birds, chickens and commercially available ensiling strains.

This proof of concept study has illustrated one approach to finding a strain of LAB capable of detoxifying DON in fermented animal feeds. The use of LIMOX media to screen for a possible epoxide hydrolase or similar enzyme was ineffective and the use of DON itself is warranted for future initial screening tests (Völkl *et al.*, 2004), unless a rapid assay for de-epoxidase is developed. So far no other research has been able to elucidate a microbe able to deactivate DON in fermented feeds, even though some groups have found some bacteria to eliminate DON under other conditions such as *in vivo* (Kollarczik *et al.*, 1994) and *in vitro* incubations with aquatic isolates (Völkl *et al.*, 2004, Ito *et al.*, 2012). In general, DON is highly resistant to modification by non-anaerobic strains, which may be linked to the main function of DON. DON is stable under the conditions *in planta* where the plant is unable to degrade the toxin and simply conjugates it to a sugar as DON-3-glucoside for safe storage using the UDP-glucosyltransferase enzyme (Poppenberger *et al.*, 2003). Since DON does not affect LAB growth and is not removed from broth, there is

evidence to suggest that a DON detoxification mechanism is absent, perhaps due to the lack of niche overlap.

LAB are present as symbionts in the GI tract of birds at levels that can influence the health of the individual (Fuller, 1973). The LAB community develops following colonisation throughout the bird's lifetime of LAB sourced from food and nesting material, water and parental carryover (Baele, Devriese *et al.* 2001). Of the many LAB species likely to be found in such an environment, it is interesting that DUN1 and PAYG5 should have comparable growth characteristic to the commercially marketed fermented feed strains. This highlights the potential for further isolation of microbes from wild birds in the future. Even though none of the strains affected levels of DON *in vitro,* the fact that these strains produced sufficient carboxylic acids to inhibit mould conidia germination by lowering silo and conidia cytoplasmic pH (Alakomi *et al.*, 2000), means they could factor in a fermented feed preparation for poultry. The benefits of producing high levels of carboxylic acid also include the acid hydrolysis of complex sugars and a potential interaction with DON. With a greater source of sugars in fermented grain feeds, it is possible that a sufficient amount of lactic acid is produced to give the effects on DON recovery found in this study. Conversely to this last point, Mansfield *et al.* (2005a) found conditions in corn silage to have little effect on reducing the levels of DON found in the starting material, and a similar silo experiment is required to confirm the results presented here.

This DON-lactic acid interaction is worthy of further investigation for two reasons. Firstly, it is possible that a lactic acid-related modification is being made to DON, which depending on the type of transformation, may be a method of bioremediation in its own right. Secondly, the fact that biopreserved feeds tend to have high levels of lactic acid

may mean that any DON testing procedure should bear in mind the mechanism of lactic acid interaction and neutralisation by NaOH may result in an underestimation of DON present. It will be interesting to investigate whether the interaction is related to that between propionic acid and sodium metabisulphite (Dänicke *et al.*, 2009). As a side point influence of binding agents on mycotoxin testing has already been evaluated in an EU report (Kolossova *et al.*, 2009), a similar approach may be needed for fermented material, especially taking into account the increased interest in MT fate within fermented feeds (Pereyra *et al.*, 2008, Keller *et al.*, 2013)

The lack of *in vitro* DON binding or metabolism by LAB isolated from the crop and gizzard does not equate to birds not having the ability to detoxify DON *in vivo.* Young and co-workers (2007) found chicken microbes in the large intestine to be responsible for converting DON to DOM-1 (de-epoxide form) and it is plausible that a different group of bacteria exist in wild birds performing a similar task. Having the ability to undertake such a biotransformation is of little consequence to the bird's health as absorption of DON and liver damage would occur before reaching the lower intestine. According to Yunus *et al.* (2010), Chickens have a much-reduced absorption of DON compared with other animals with only 0.044% of ingested DON found in the plasma. This amount is rapidly excreted and a degree of de-epoxidase activity also occurs in a minority of the birds suggesting uptake of the DOM-1 form in the lower intestine following microbial biotransformation. A similar phenomenon may occur in the birds studied here with any resistance to cereal-borne MT a function of reduced absorption rather than microbial transformation. Further work is required to understand the effect of MTs on wild bird populations, a completely unexplored research area. The isolation of LAB from the fore-gut was aimed at finding a

more transferable isolate than from the very anaerobic condition in the lower intestine which would not yield isolates capable of growth and competition in fermented feeds.

The current study focusses on LAB in part due to their omnipresence in fermented animal feeds, but also related to their GRAS (generally recognised as safe) status and ease of culture/isolation. This does limit the search for a DON degrading bacterium somewhat, and had LIMOX media been used with bulk bacterial assemblage isolates from the GI tract of birds or soil for example, it may have selected for better candidates. This approach has been explored by Ito and colleagues (2012) who were able to find DON-degrading bacteria employing in house produced and purified DON as the sole carbon source, which is always going to be the best option providing the safety concerns can be answered.

This first experimental chapter entertains the idea that LAB may be able to both produce a quality fermented feed whilst being instrumental in DON degradation. However, whilst LAB have been found to bind trichothecene MTs such as DON, ZEA and T2 toxin, there is a paucity of evidence suggesting anything more significant such as metabolising MTs into lesser toxic elements. Probiotic strains of LAB have been shown in the past to be ineffectual bio-degraders of trichothecenes (including DON) *in vitro,* which is in agreement with the current data (Böhm *et al.*, 2000). The results here in a 48 h growth experiment show little evidence of binding, metabolism or both, but it must be considered that these are processes could occur over different timeframes. Some workers have found that MT binding in LAB occurs most frequently during exponential growth phase and that the degree of binding is partially reversible with MT being released back into the medium over time (El-Nezami *et al.*, 2004). Since binding has been found to be a reversible process, employing this property to detoxify ensiled grains would be highly

questionable due to the likelihood of desorption on GI transit (*i.e.* no likely difference in toxicity). Moreover, in a fermented feed scenario the presence of LAB producing LA/AA may actually inhibit the growth of co-occurring microbial assemblages with DON biotransformation capacity and thus relying on LAB alone may be counterproductive in terms of DON detoxification. However, in terms of the inhibition of spoilage mould LAB have been shown, both in the current investigation and in the literature, as extremely effective and therefore when considering the safety and quality of fermented feeds as a whole they still have an important role to play. Interestingly, DUN1 and PayG5 produced comparable inhibition to that of the commercially available silage additives linked to the suite of acidic secondary metabolites produced, especially acetic and propionic acids.

The lack of DON metabolism also equates with the equivocal result found with the LIMOX sole carbon source experiment. This could be due to a lack of enzyme present in LAB that catabolise or co-metabolise this type of molecule. LAB thrive on media rich in water soluble carbohydrates, less so on carbon sources beyond the realm of homolactic and heterolactic fermentation of hexoses and pentoses. They consequently have a low capacity for direct biotransformation, although co-metabolism via Mailliard reactions may occur where exuded proteins and sugars deactivate DON indirectly. An ability to biotranform DON by *Aspergillus tubingensis* was found by He and co-workers (2008) to be due to the addition of H_2O (hydration) with the putatively described epoxide hydrolase responsible. Epoxide hydrolase is the same enzyme responsible for the conversion of LIMOX to limonene and hence would be a useful enzyme to find in LAB, although gene-sequence led research suggests that LAB lost their epoxide hydrolase genes during evolution, as bacteria have a bias towards deleting genes no longer needed (Van Loo *et*

al., 2006). The only bacteria able to biotransform LIMOX to limonene-1,2-diol with soluble epoxide hydrolase was *Corynebacterium* sp. C12, a bacteria species originally isolated using an epoxide (the hazardous cyclohexene oxide) as a sole carbon source (Carter and Leak, 1995).

3 The effect of different bacterial inoculants, chemical preservatives and heat treatment on crimped grain silage fermentation and deoxynivalenol recovery

3.1 Aims and objectives

To evaluate the effect of different CG treatment strategies on the reduction of DON in experimental silos using a validated chromatographic detection method, determine important biochemical properties of the fermentation and verify if these metabolites have any effect on DON stability *in vitro.*

3.2 Introduction

Fusarium head blight (FHB) is a grain disease prevalent in temperate cereal growing regions with *Fusarium culmorum* and *F. graminearum* the main pathogenic moulds responsible. During the process of infection several MTs including DON are produced by these moulds to aid colonisation of the plant (Poppenberger *et al.*, 2003). Levels of DON can reach toxic levels when prevailing weather conditions are wet during crop anthesis facilitating spore egress, a phenomenon not always preventable with topical application of fungicides or choice of resistant cultivars (Langseth and Elen, 1997, Mansfield *et al.*, 2005a). Such episodes were observed in the 2008 UK harvest where levels in certain cereals precluded the incorporation into food and feed with associated financial losses (Day, 2009). To minimise the effect on livestock a range of chemical and biological additives are being developed which have the potential to bind and degrade DON under specific conditions (Dänicke *et al.*, 2009, Völkl *et al.*, 2004). In the current investigation, the process of ensiling is evaluated as having the potential for bioremediation of contaminated material.

CG silage feeds are an integral part of temperate agriculture where growing seasons can be disrupted by climate and a locally produced feedstock high in nutrients is desirable. Grain is harvested with a moisture content of around 40% and ensiled with biological or chemical preservatives prior to storage in airtight silos (Adesogan *et al.*, 2003). The main purpose of these additives is to reduce the pH to the critical 3-4 level where bio-deterioration and microbial respiration are curtailed (Virtanen, 1945). Whether DON levels are affected by the conditions of ensiling, in terms of microbial interaction and fermentation chemistry, forms the main basis of the current study. In addition, the measurement of DON-3-glucoside (D3G) in crimped feed was investigated.

Several different strains of lactic acid bacteria (LAB) are trialled as biological additives following crimping and compared with a combined chemical preservative (Crimpstore) and heat treatment strategy for the ability to assist in the production of an efficient fermented feed with the potential for reduction of DON. The efficiency of the ensiling process is characterised by measuring several biochemical parameters such as organic acid and soluble sugar concentration, and related these to the viable counts of yeast and LAB.

3.3 Materials and methods

3.3.1 Spiking with DON and ensiling of crimped barley

Barley (Westminster, 38.40±0.37% moisture) was harvested and crimped in July 2011 in the south west of the UK prior to treatment with the chemical preservative Crimpstore[(R)] 2000 S (an additive containing a mixture of formic acid (43%), propionic acid (10%), ammonium formate (30%), and benzoic acid (2%) in water, Kelvin Cave Ltd., UK) at a rate

of 4 ml/kg or inoculated with different commercially available or in-house isolated lactic acid bacteria (identified with 16S rRNA identification: see Chapter 4) at a rate of (10^5 CFU/g moist grain) in 50 ml polystyrene containers (Fisher, Loughborough, UK) containing approximately 50g of grain (exact weight of each sample taken for calculations). This was followed by spiking with DON standard in H_2O (Sigma Aldrich, Poole, UK) at 250 µg silo^{-1} (concentration of 5 µg/g CG), tightly closing the lid and incubating at 20 °C for 21 d in the dark. Silos spiked with DON but without additives served as naturally fermented samples and spiked samples kept frozen for the same period served as unfermented controls allowing comparison for the treated samples. In addition, samples heat treated prior to spiking (100°C for 1 h in hot air oven) served as semi-sterile incubations. The nutritional proximate analysis results for the barley were as follows: lipid 0.92%, ash 1.36%, protein 6.76%, carbohydrate 53.00% and moisture 38.40% (analysis undertaken by Liz Preston, Food and Nutrition, Plymouth University)

3.3.2 Extraction and HPLC analysis

Ninety five ml of DPBS (plus 5 ml H_2O from pH determination, see below) was added to the entire silo contents and blended for 2 min on full speed (Waring Blender, USA) and 10 ml of supernatant was passed through a Whatman 113 filter paper (according to sample preparation and chromatography guidelines provided by Rhōne-Biopharm Ltd). This filtrate was then centrifuged at 5000g for 10 min and passed through a 0.22 µm syringe filter (Millipore). Supernatant (2 ml) was then passed drop-wise through a DONprep$^{(R)}$ immunoaffinity column (Rhōne-Biopharm Ltd, Glasgow, UK) and the column rinsed with 5 ml HPLC grade H_2O, also dropwise (1 drop/s). All H_2O was expelled and 1.5 ml of HPLC grade methanol (MeOH) was passed drop-wise through the column with 1 backwash

included using the affixed syringe. This eluent was collected in a glass test tube and blown down to dryness with compressed nitrogen and re-suspended for 30 s using a vortex mixer with 1 ml 5% (v/v) MeOH in water. The extracts were passed through a 0.22 μm syringe filter for HPLC (Dionex UltiMate® 3000, Thermo Scientific). The HPLC gradient program was as follows; 0 min 5% MeOH, 20 min 5% MeOH 1% ACN, 21 min 50% MeOH 1% ACN, 25 min 50% MeOH 1% ACN, 26 min 5% MeOH 1% ACN to 30 min. Fifty μl of sample was injected and the DON was eluted at 23 min. The C18 column (Aquasil C18, 150 mm x 4.6 mm, 3 μm, standard, Thermo) and C18 guard column was kept at 25 °C and a UV detector set at 220 nm (5 nm bandwidth). The peak area was used to compare between DON levels in the treatments compared with the frozen time zero DON-spiked control. The crimped barley used in this study contained zero natural DON contamination.

3.3.3 Validation of DON-specific IAC columns for use with D3G and crimped feeds including verification of optimum analyte loading concentration, and effects of organic acid concentration and temperature on D3G/DON stability

The capacity of the DONprep[(R)] immunoaffinity columns for both D3G and DON was determined by passing various concentrations of compound through the column and measuring the concentration before, in the initial eluate and in retained fraction using HPLC and following the manufacturer's protocol as mentioned previously. HPLC analysis of DON/D3G concentrations (in PBS) following the sequential addition of lactic acid or following heating enabled further characterisation of DON/D3G stability within the silo environment. Additional HPLC studies were undertaken to determine the effect of increasing concentrations of lactic, acetic and propionic acid on *in vitro* DON and D3G stability (50 μg/ml H_2O) by sequential 50 μl HPLC injections and replacement additions of

30, 300 and 3000 mM acid stocks and calculating the adjusted peak area according to the reduced DON concentration. The effect of heating at 50, 75 and 100 °C was also determined.

3.3.4 Determination of silage integrity

On mini-silo opening 5 ml of H_2O was mixed with 1 g fermented CG for determination of pH (Fisher Accumet) and added back to the silo contents. Following addition of 95 ml PBS and blending, a 1 ml sample was serially diluted in PBS and plated on MRS (de Man, Rogosa and Sharpe), RCA (reinforced clostridial agar, samples heated to 70 °C for 5 min to confirm presence of resistant spores and incubated for 48 h anaerobically at 37 °C and CODA chloramphenicol/ oxytetracyline dextrose agar, 25 °C) agar for enumeration of lactic acid bacteria, clostridia and yeast/moulds respectively (moulds were distinguished from yeasts by their furry appearance). In addition, a 250 µl sample was deproteinated with 250 µl 0.5 M perchloric acid and then neutralised with twice the sample volume of dipotassium carbonate (1 M), centrifuged at 10000g, spiked with 20 µl of 7% H_2SO_4 and 20 µl was injected into an HPLC (Dionex) for the presence of lactic, acetic, propionic, butyric acids and maltose, fructose, glucose and ethanol (Agilent H^+ column, 0.4 ml/min 5 mM H_2SO_4 with Shodex (Japan) RI detector) based on the method published by Niven *et al.* (2004)

3.4 Results

3.4.1 Validation of IAC columns for use with D3G and crimped feeds and effects of organic acid concentration and temperature on D3G/DON stability
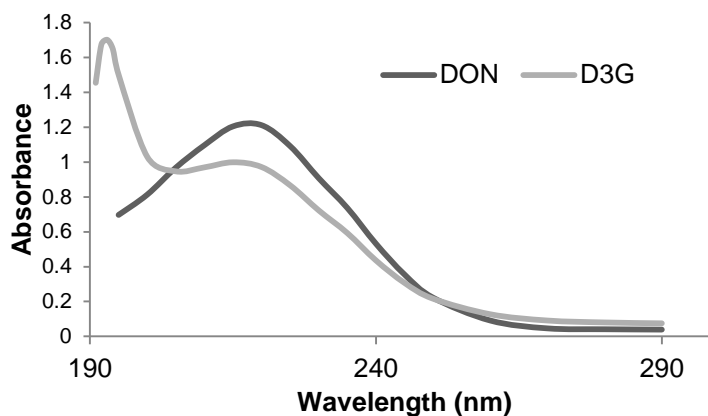
Figure 3.1. The UV absorption spectra of DON and D3G (5 µg/ml). Samples suspended in far-uv grade ACN.

Using a wavelength of 220 nm for DON and D3G was found to be appropriate when using HPLC-UV following spectrophotometric analysis of standards suspended in far-UV grade ACN and analysed with a UV spectrophotometer (Helios Beta, Unicam, UK, Figure 3.1). Extracted samples processed with IAC could not be analysed with this technique due to the poor sensitivity attributed to cross-contaminants in the column matrix, hence the need for HPLC-UV. The DONprep IAC tested did show an affinity for D3G, although the loading capacity of the column was slightly lower compared with DON (Figure 3.2). The optimum loading of the column, where approx. 100 ±10% of the analyte loaded in 1ml was actually measured following the IAC manufacturers protocol, was between 0.5-2 µg/ml for both DON and D3G. Below this concentration range the proportion recovered was reduced, perhaps due to the analyte not eluting completely from the column.
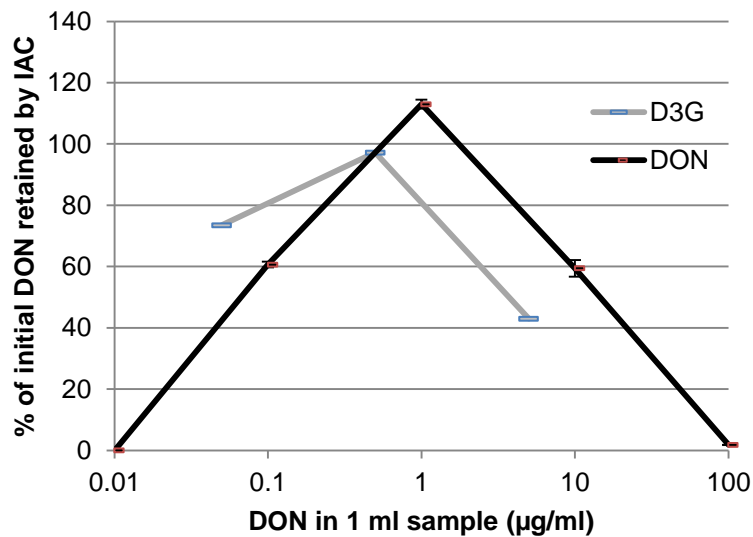
Figure 3.2. Comparison of immunoaffinity column capacities for DON and D3G. 1 ml of a range of concentrations were passed through different columns, eluted with methanol and re-analysed with HPLC. Mean of 2 replicates shown with SE.
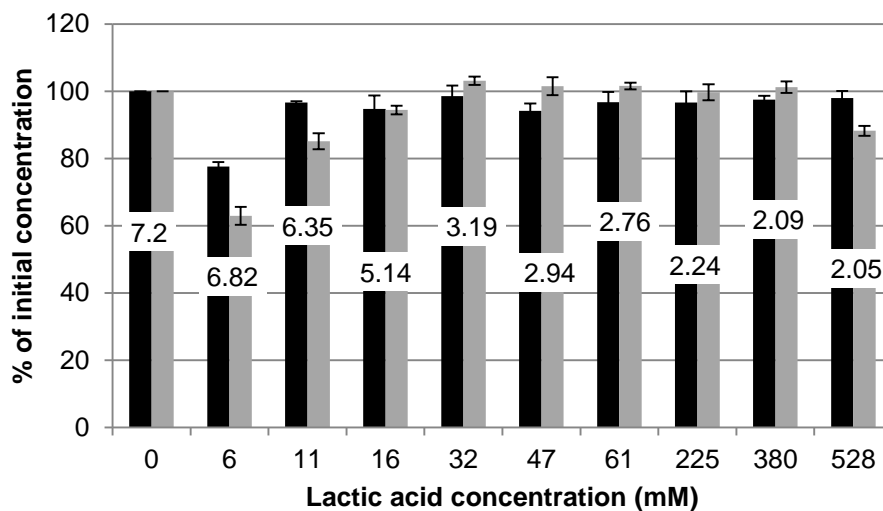
Figure 3.3. D3G (black) and DON (grey) stability in DPBS following serial additions of lactic acid (mM). The mean of duplicate runs is shown with the SE. The pH is shown for each concentration.
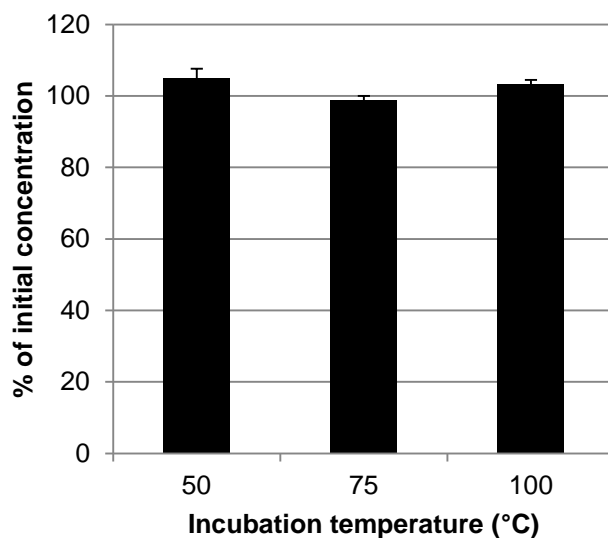
Figure 3.4. The effect of heating on the stability of the glucosidic bond in D3G. Data shown as percentage of initial concentration (25 °C) prior to heating for 10 min at 50, 75 and 100 °C. The mean of duplicate treatments is shown with the SE.
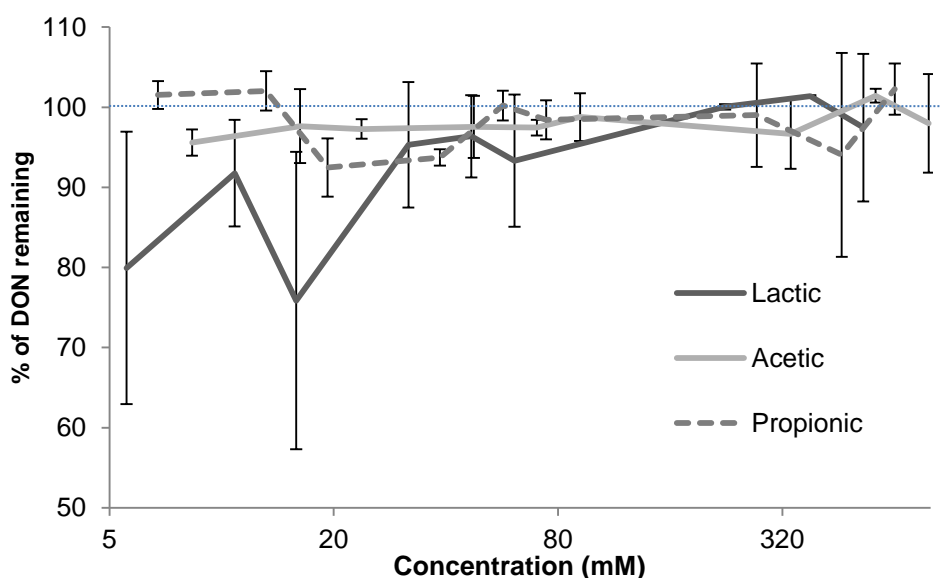
Figure 3.5. The effect of lactic, acetic and propionic acid concentration on DON stability incubated in PBS at 25 °C. Data shown as percentage of initial measured concentration in triplicate with SE.

Treatment of D3G and DON with lactic acid or heat up to 100 °C did little to reduce the detection and thus amounts of DON or D3G (Figure 3.3, 3.4) and reaffirming the stable nature of these compounds. Treatment of DON with propionic or acetic acid gave a similar response to lactic acid (Figure 3.5). The drop in response between pH 7.2 and 6.35 suggests a potential shift in the ionic status leading to a modulation in UV absorption or

modifying the interaction with the c18 matrix rather than any degradation of compound as the higher concentrations yielded close to 100% recovery. The highest concentration tested of 500 mM equates to silo levels of 360, 240 and 296 mg/g CG of lactic, acetic and propionic acid respectively, which corresponds to 5, 14 and 28x that found naturally in the experimental silos (Figure 3.5). This indicates that organic acids themselves are not likely to be instrumental in DON remediation and conversely may restrict growth of those organisms with potential DON metabolism capacity.

3.4.2 Levels of DON following ensiling
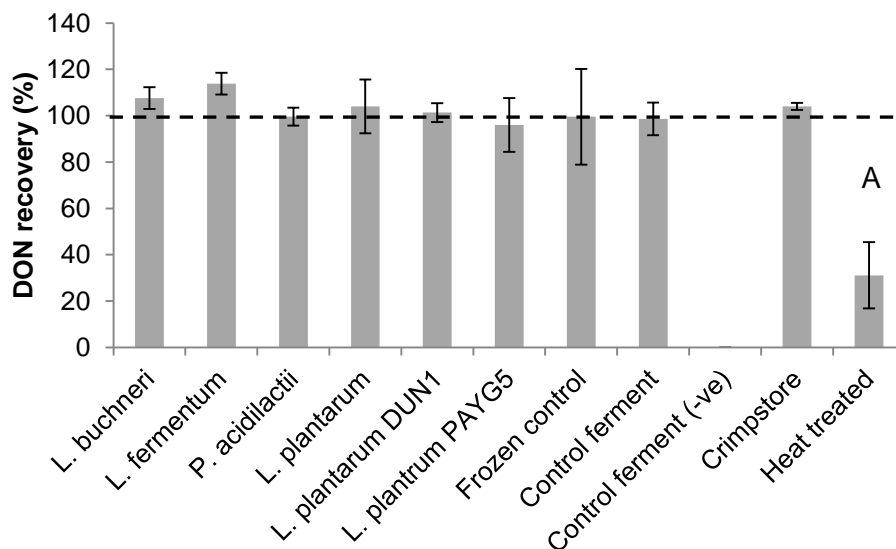
Figure 3.6. Percentage recovery of crimped grain silos spiked with DON (5 µg/g) following a 21 d incubation at 20 °C either with the addition of lactic acid bacteria, the chemical preservative Crimpstore®, no addition of treatment or/and DON, frozen for duration control (5 µg/g) or heat treated prior to addition of DON. Mean percentage of unfermented frozen control silos in triplicate shown with SE and significantly different treatments labelled with A (p≤0.001)

A 21 d mini-silo CG spike and recovery experiment was carried out with DON in order to determine if any farm-relevant bacterial additives or chemical treatments were able to reduce DON and render the CG safer for animal consumption. Interestingly, the levels of DON recovered from the spiked CG was not significantly affected by any of the lactic acid bacteria, chemical preservative or natural fermentation treatments, yet the heat treated

CG silos yielded a significant 68.8 ± 14.4% decrease ($p<0.001$, ANOVA) in DON recovery compared with the other treatments (Figure 3.6). The grain was heat treated prior to ensiling and spiking with DON, therefore a biological or chemical component was likely responsible for this phenomenon. Whilst it may be thought to be an oversight not to spike with DON and then heat the material, DON was shown to be resistant to heat and this treatment was initially designed to be a reduced inocula control rather than a realistic CG ensiling scenario. None of the samples yielded any measurable amounts of D3G indicating *de novo* conjugation in planta did not occur in the period between spiking with DON and fermentation. Recovery in the frozen control was 99.6 ± 20% when considering the total amount of DON spiked as 250 µg/silo (therefore bypassing the need for dry weight). LOD was 0.01 µg/ml and 0.035 µg/ml for DON and D3G standards respectively.

3.4.3 Fermentation characteristics

Numbers of LAB and yeasts In the mini-silos following incubation for 21 d was similar between all LAB strain treatments with DUN1 (*L. plantarum*) producing the lowest yeast counts (Table 3.1), which corresponds to the highest measured lactic and acetic acid content. There was also correlation between levels of lactic, acetic and ethanol (Figure 3.7, Table 3.2) indicating the development of a bi-phasic fermentation dominated by homofermentative then heterofermentative LAB with acid tolerant yeasts possibly forming additional viable populations. This demonstrates how fermented feeds have a degree of microbial and metabolic succession governed by nutrients, anaerobiosis, acidification and diversity of initial inocula and is supported by comparing with the time series data (2 and 4 weeks) presented in Chapter 5. The measured pH was approximately 4 in all cases, indicating water soluble carbohydrates (WSC) levels were initially

favourable for swift homolactic fermentation. These measured properties suggest the fermentation is of high quality and a reasonable representation of actual CG silo processes, although this is related to a zero-decrease in the recovery of DON.

Crimpstore® did not prevent biological fermentation outright at the concentration used, yet the yeast count was dramatically reduced. The amount of LAB enumerated suggests that Crimpstore® may enable a stable population of LAB to develop over the 3 weeks, albeit to a lesser extent as supported by the reduced utilisation of WSC including maltose and fructose. Given more time a greater colonisation by LAB may occur with the Crimpstore®, providing WSC levels are sufficient.

The heat treated samples produced the largest counts on MRS agar with appreciative amounts of propionic acid present suggesting that the community deviates from the conventional LAB-based assemblage supported by the low level of lactic acid and high pH. In addition, the ability to withstand the heat treatment suggests the community assemblage is potentially made up of heat resistant species. Levels of glucose and maltose were similar to the LAB fermentations yet fructose was absent from the silo extract, suggesting the bacteria were chiefly exploiting fructose for energy. There were zero yeast counts on CODA illustrating how heat treating reduces the yeast population either directly by heat effects or indirectly by matrix modification, pH and inhibitory secondary metabolites secreted by colonising bacteria. The presence of propionic acid was correlated to the only drop in DON levels (Table 3.2, Figure 3.8) which makes the select microbial community present especially interesting and the agent responsible worth elucidating. Clostridia spore counts were below 1 CFU/g CG for all treatments supported by the lack of butyric acid, and viable mould spores were at a similar low level.

Table 3.1. Measurements of fermented crimped grain feed integrity after 21 d incubation at 20 °C following heat treatment (100 °C, 60 min) or addition of biological and chemical additives. The LSMEAN of triplicate silos is shown with the SE and p-value (1 way ANOVA). Treatment parameters sharing the same letter do not differ significantly (*p*<0.05).

	L. buchneri	*L. fermentum*	*P. acidilactii*	*L. plantarum*	*L. plantarum* DUN1	*L. plantarum* PAYG5	Control fermentation	Crimpstore®	Heat treated	SE	*p*-value
Maltose[1]	0.5	0.4	0.4	1.0	1.0	0.7	0.2	11.8^a	0.8	2.4	<0.001
Glucose[1]	5.6	5.6	6.8	7.4	7.8	6.0	5.3^b	2.7^b	8.1^a	0.8	<0.001
Fructose[1]	2.3	1.5	1.8	2.9	3.3	3.1	3.0	64.8^a	0	8.7	<0.001
Citric[1]	3.7	5.5	3.7	5.6	5.7	3.4	5.3	12.0^a	1.2	1.7	<0.001
Lactic[1]	66.7	65.1	58.5	67.3	77.8	64.5	70.6	24.6^b	2.7^b	9.3	<0.001
Acetic[1]	12.2	7.4^b	11.5	14.7	17.3^a	10.8	9.9	4.0^b	1.6^b	2.5	<0.001
Propionic[1]	0	0	0	0	0	0	0	10.5^a	8.2^a	2.6	<0.001
Ethanol[1]	35.6	39.4	35.4	40.1	51	41.8	48	22.6	29.2	14.3	0.388
MRS[2]	6.02	5.62	5.58	5.38	5.62	5.58	5.93	5.09	6.4	0.5	0.125
COSA[3]	3.38	3.32	3.57	3.49	3.04	2.78	3.39	0.98^b	Nil	0.5	0.003
pH	4.05	4.17	4.12	4.09	4.00	4.10	3.96	4.02	5.65^a	0.5	<0.001

[1]Water soluble carbohydrates and metabolites (mg/g crimped grain)
[2]$\log_{10}$ viable LAB CFU/g crimped grain
[3]$\log_{10}$ viable yeast/mould CFU/g crimped grain
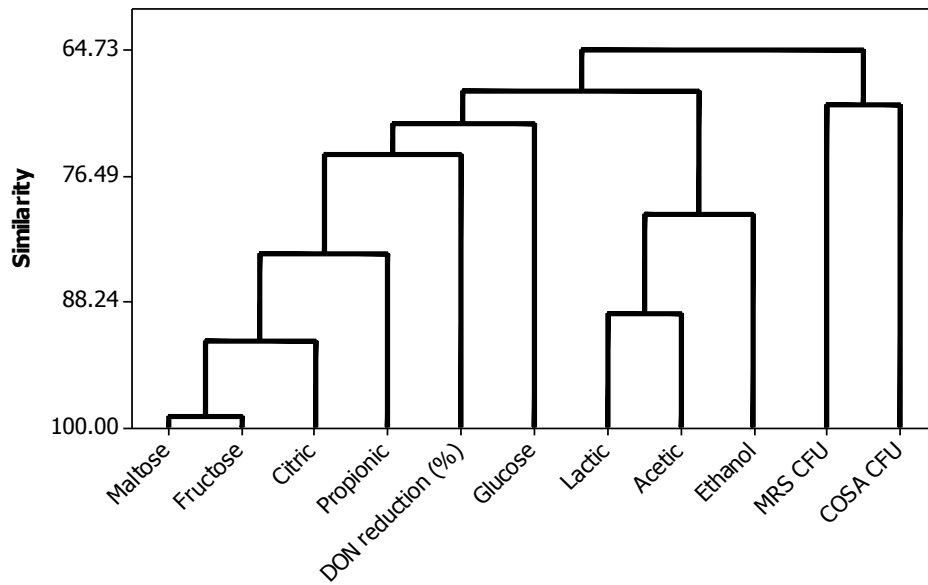
Figure 3.7. Dendrogram illustrating the correlation between parameters measured in individual silos (correlation coefficient distance, single linkage). Adjoined parameters are the most similar.

Table 3.2. Pearson correlation coefficients of parameters measured following incubation of crimped barley with different treatments. Somewhat significant interactions (<0.01) are shaded grey. N = 33

	Lactic	Acetic	Citric	Propionic	Ethanol	Maltose	Glucose	Fructose	MRS CFU	COSA CFU	% DON reduction
Lactic		0.788 <0.001	0.042 0.836	-0.785 <0.001	0.601 0.001	-0.417 0.030	0.078 0.700	-0.389 0.045	-0.331 0.091	0.295 0.136	-0.717 <0.001
Acetic			-0.053 0.795	-0.686 <0.001	0.420 0.029	-0.413 0.032	0.369 0.058	-0.393 0.042	-0.342 0.081	0.148 0.462	-0.511 0.006
Citric				0.337 0.086	-0.004 0.984	0.804 <0.001	-0.622 0.001	0.840 <0.001	-0.417 0.031	-0.200 0.318	-0.457 0.016
Propionic					-0.463 0.015	0.676 <0.001	-0.230 0.249	0.653 <0.001	0.015 0.940	-0.348 0.075	0.490 0.010
Ethanol						-0.319 0.105	0.194 0.333	-0.309 0.117	-0.265 0.181	-0.039 0.847	-0.166 0.404
Maltose							-0.630 <0.001	0.979 <0.001	-0.161 0.423	-0.263 0.186	-0.112 0.579
Glucose								-0.710 <0.001	0.168 0.402	0.014 0.944	0.431 0.025
Fructose									-0.218 0.275	-0.252 0.205	-0.157 0.434
MRS CFU										0.397 0.040	0.265 0.181
COSA CFU											-0.203 0.310

87
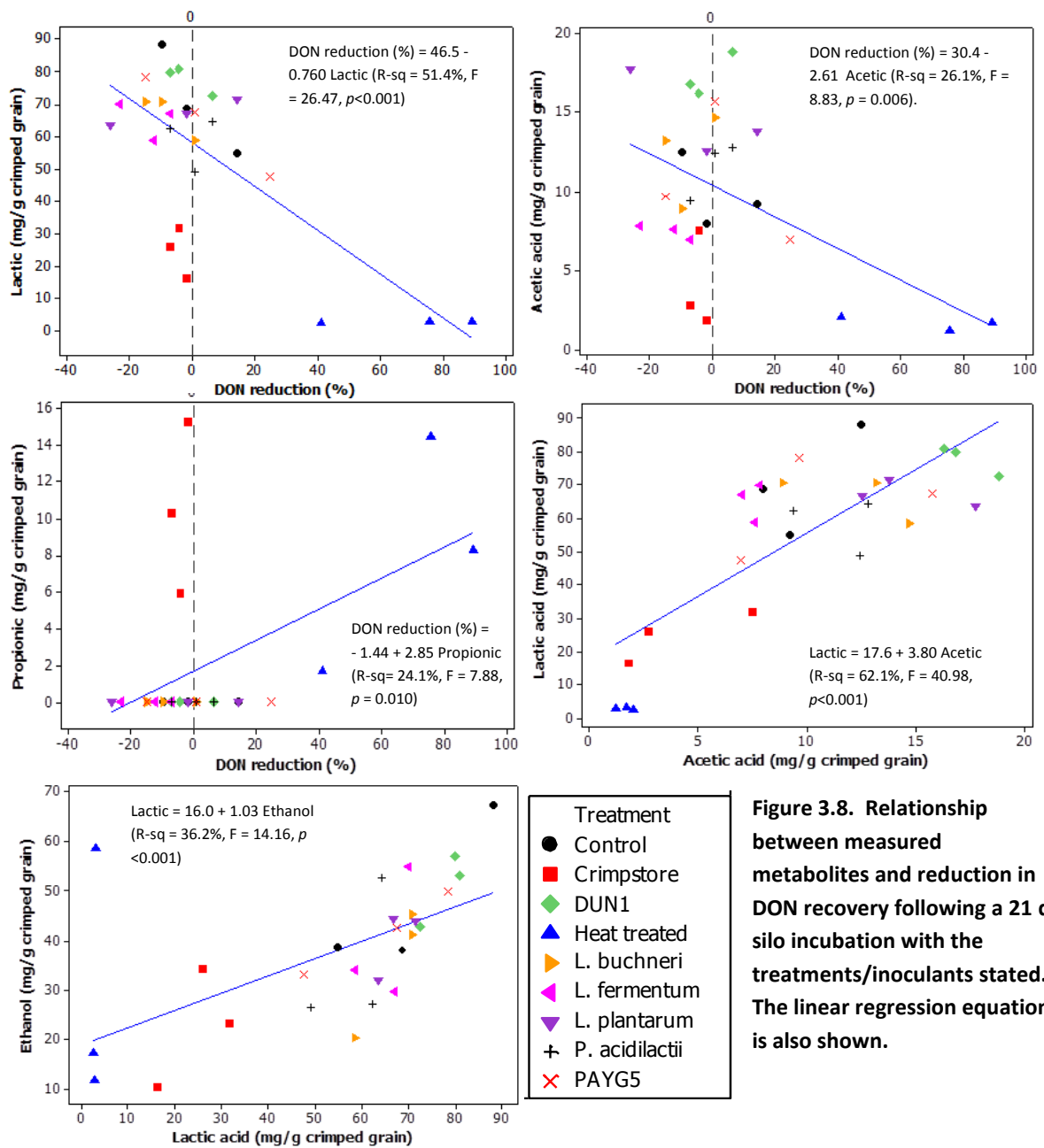
Figure 3.8. Relationship between measured metabolites and reduction in DON recovery following a 21 d silo incubation with the treatments/inoculants stated. The linear regression equation is also shown.

3.5 Discussion

DON contaminated grain has the potential for significant reductions in livestock performance and possible toxicosis (D'Mello *et al.*, 1999). Extensive research has already been undertaken regarding the transfer of DON through various levels of food and feed preparation (Kostelanska *et al.*, 2009, Binder *et al.*, 2007, Simsek *et al.*, 2012), yet very little attention has been directed towards the field of fermented feeds as a route of DON bioremediation. This is the first study to investigate DON in the context of CG feeds,

where the high moisture content and the lack of routine MT testing due to the in-house nature of the feed requires a different approach to dry commodity feeds.

Considering the current results, the channelling of contaminated grain into the conventional fermented feed process in order to reduce the levels of DON should however be undertaken with caution. As illustrated in the current study the LAB-based fermentation or chemical preservative approach to ensiling yields a consistent product after 3 weeks with an appreciable degree of biochemical stability, yet levels of DON are found to be as equally stable. This finding is in common with a range of other studies where the stability of DON is highlighted in a wide variety of matrices and commodities, including maize silage where levels of DON actually increased following fermentation (Keller *et al.*, 2013, Pereyra *et al.*, 2008). Mansfield and co-workers (2005b) conversely found levels of DON to decrease within ensiled maize, an effect that could not be attributed to physical or chemical properties of the material. Instead the researchers suggested that a microbial element was responsible within the silo and envisaged that post-harvest control of DON could indeed be possible and even speculating that these microbial agents could extend their beneficial DON degradation effects to within the rumen of cattle.

The ensiled grain samples produced no detectable amounts of D3G, even though the barley used for crimping did possess this ability (chapter 5). The IAC-columns employed for clean-up had sufficient cross-reactivity for D3G, as was confirmed by Veršilovskis and co-authors (2011). It may be concluded that the process of crimping and ensiling is unlikely to convert DON to D3G within the ensiling timeframe. It is likely that the grain quickly loses the ability for metabolic conjugation during the initial development of

fermentation conditions in the silo. Further work is required to assess the potential de-conjugation of D3G under fermentation conditions, however exposure to acids and heat did not affect D3G measurements *in vitro,* a result supported by recent studies by Vendl *et al.* (2010) and Berthiller *et al.* (2011) who suggest only enzymatic hydrolysis could cleave the glucoside residue in a cereal context and heat/acid hydrolysis at pH 1.3 were ineffective. An exciting result by Berthiller *et al.* was that *L. plantarum* was shown to possess the ability for this hydrolysis in media and hydrolysed 62% of D3G over 8 h at 37 °C. Since *L. plantarum* is endemic to fermented feeds, it can be hypothesised that D3G may have a short half-life within a silo. This is investigated in chapter 5.

In the current study, the only treatment to yield a reduction in levels of DON was the heat treated CG treatment, which also surprisingly had the highest bacterial count recorded on MRS agar, yet also had the highest pH (5.65). A homolactic/heterolactic LAB-based fermentation phase yields a swift drop in pH suggesting the heat treated silos are still in the aerobic phase of ensiling, characterised by a high pH, bacterial counts and WSC concentrations. It is worth mentioning that without a rapid drop in pH, the residual carbohydrases and proteases are unhindered to strip the feed of its nutritive value (Virtanen, 1945). Curiously, there is a distinct possibility that this higher pH facilitated the enzymatic biotransformation or binding of DON within these silos, although a cause and effect explanation is complex, although it is likely that any plant enzymes would have been denatured on heating leaving viable bacteria the sole source of such an agent.

Though the heat treatment deviates from the normal ensiling process with the possibility of the feed being inferior nutritionally, of low aerobic stability and possessing a modified matrix for bacterial growth, the resulting bacterial assemblage was associated with a

significant decrease in DON and is therefore worthy of further investigation in terms of the development of silage additives. Further work is presented in the following chapters which involves the isolation of potential candidate bacteria from these silos with selective media and determining the efficacy of DON degradation *in vitro* and in naturally DON-contaminated grain produced from wheat and barley inoculated under greenhouse conditions with *F. culmorum.*

The results presented in the current chapter contribute to the overall understanding of DON degradation in field-borne and fermentation scenarios. It is interesting that the bacteria able to metabolise DON were originally derived from the spikelets of harvested barley. It could therefore be hypothesised that the metabolic capacity to degrade DON stems from the ecological need to compete for compartmentalised resources. The concept of reducing DON in naturally contaminated grain of wheat or barley using a DON-degrading bacterium derived from the phyllosphere of the wheat or barley itself has been recently explored by Ito and co-workers, who isolated a *Marmoricola* sp. from the heads of wheat and applied it to DON-contaminated material, yielding a 66% decrease in DON (2012). The same group has more recently isolated a *Sphingomonas* sp. from lake water which had DON-catalysing capacity via a bacterial P450 enzyme system, illustrating that biodegradation pathways of MTs could be both employed for reduction of FHB effects *in planta* and in harvested commodities (Ito *et al.*, 2013).

CG feeds form an important method of forage preservation in many regions of the globe where short growing seasons and disruptive weather patterns preclude conventional dry grain animal feeds and can cause increased incidence of FHB and DON contamination. Whilst lactic acid fermentative and chemical preservation modes yield a consistent feed,

according to the results presented the level of DON in harvested grain is likely to remain unaffected following ensiling. Concerning CG feeds in general, it is apparent that the addition of different LAB at sufficient inoculation rates does increase the likelihood of that strain playing a part in the overall fermentation and thus shaping the fermentation characteristics and community assemblage ecology, yet the final result can be similar to that obtained with a natural fermentation providing conditions permit. Where the strategy of adding LAB strains with desired properties has most benefit is firstly immediately following anaerobiosis; where swift colonisation, production of organic acids and reduction of pH avoids excessive aerobic loss, and secondly; when the presence of facultative heterolactic LAB such as *L. buchneri* convert lactic to acetic acid and thus further reducing the pH and inhibiting undesirable microbial activity during storage and feed utilisation (Holzer *et al.*, 2003, McDonald, 1981). For this reason and considering the DGGE results in the following chapter, it would be prudent to ensure that the obligate heterofermentative *L. buchneri* is present along with homofermentative LAB such as *L. plantarum* in all on-farm CG strategies where fermentation consistency is critical (Filya *et al.*, 2006, Arriola *et al.*, 2011). Crimpstore® achieved a presumably similar degree of long-term stability due to the very low yeast counts at enumeration and potential for greater increases in LAB and consolidation of low pH due to the amount of WSC remaining.

4 Bacterial assemblage analysis in crimped grain mini-silos leading to Isolation of potential DON-biotransforming bacteria from silos exhibiting a reduction in DON.

4.1 Aims and objectives

The objectives are to firstly catalogue the bacterial community assemblage associated with each mini-silo fermentation, specifically targeting those which exhibited a modulation in DON recovery, and secondly devise a bank of selective media and incubation criteria to isolate live bacteria from the silo sample for downstream DON-degrading applications and characterisation.

4.2 Introduction

Biological and chemical-based methods exist for the ensiling of CG for animal feeds. Both strategies involve the use of organic acids to prevent growth of spoilage organisms and preserve the nutritive value of the material without affecting the palatability or safety of the feed (Dunière *et al.*, 2013). Conversely, it is hypothesised that such treatments may facilitate certain strains of bacteria to colonise the feed and in turn reduce levels of DON by biotransformation and binding, therefore increasing feed safety and animal welfare yet further (Boudra and Morgavi, 2008). The epiphytic bacterial assemblage associated with the grain pre-harvest is likely to be very different to that of the mature CG silage as the changing biochemical parameters favour different bacterial groups with a succession of species culminating with a community of LAB able to thrive in the anaerobic and low pH conditions, such as *L. buchneri* (Holzer *et al.*, 2003). It is envisaged that chemical and physical conditions prior to ensiling, in addition to the epiphytic inoculum density and diversity, will be key to shaping the bacterial assemblage in the fermenting product (McDonald *et al.*, 1991). The results of the last chapter suggested that chemical

treatment can reduce bacterial numbers overall and heating the grain can result in large latent increases in viable counts; the identity of which will be elucidated in the current chapter.

When the silo material is mixed with LAB culture such as *L. buchneri* or *L. plantarum* at an adequate seeding density it is hypothesised that these LAB will shape the community by acting as keystone species resulting in silage with low pH and low growth of competing and harmful microorganisms. The community associated with a drop in DON recovery will be characterised using PCR-DGGE-SEQ (polymerase chain reaction-denaturant gradient gel electrophoresis-sequencing), a molecular technique based on the PCR and sequencing of the electrophoresis-separated 16S rRNA gene product and aligning it with a published bacterial gene sequence using BLAST (NCBI). DGGE is a technique developed by Muyzer *et al.* (1993) to primarily characterise the complex bacterial assemblage of seawater and any successional changes induced by fluctuations in environmental factors. Rather than enumerating viable bacteria on a range of selective agar to gain an insight into the microbiological community, the genomic content of the sample is analysed to identify both cultivable and incultivable bacteria species, therefore potentially yielding a far greater understanding of bacterial succession in CG, a subject only previously investigated using cultivation techniques (Olstorpe *et al.*, 2010b). Identification of bacterial groups present in each CG treatment allows a directed isolation strategy to be formulated with selective media and incubation treatments leading to storage of the live strains for further molecular analysis and DON-degradation experiments. DGGE was recently employed to great effect by Li and Nishino (2011) concerning the monitoring of bacterial community succession within whole crop maize bunkers inoculated with

different LAB. The authors were able to identify several dominant species of bacteria identified in maize (*L. acetotolerans, L. panis, Weissella cibaria*) and Italian ryegrass silage (*Erwinia persicina, Pantoea agglomerans*) for the first time using DGGE (Li and Nishino, 2013).

The effectiveness of an inoculating strain to rapidly colonise the matrix and become the dominant entity responsible for the integrity of crimped wheat grain has been undertaken previously by Adesogan and co-workers (2003), who found *L. buchneri* to be the most promising LAB in terms of aerobic stability, although Crimpstore; the chemical based alternative, was found to yield the least DM losses overall. Olstorpe and colleagues (2010b) evaluated the microbial diversity of CG silage in Sweden following different inoculation treatments and observed significant inter-farm differences in assemblage succession which was related to wide variations in silage integrity.

There is a current paucity of studies related to the modulation of MT in CG, let alone studies attributing reductions in DON to microbial community components within the fermented material. Some investigators have noted reductions in certain MT from initial levels to that in mature maize silage (Boudra and Morgavi, 2008), but as yet no research has been published which takes the next step of identifying the biological agents responsible and developed them as novel silage additives. This could be hypothesised as being a more effectual method of finding DON-degrading bacteria that screening a bank of organisms for a candidate which may not actually be applicable due to the requirement of the agent to proliferate and exist within the silage matrix. Yu *et al.*(2010) successfully used PCR-DGGE to guide the selective isolation of DON-transforming bacteria in chicken digesta using a range of antibiotics and found 10 isolates from 4 bacterial groups able to

transform DON to DOM-1 when grown and sub-cultured in specific media. Whether these bacteria could be applied to contaminated feedstock for fermented feeds or prophylactic gut seeding has yet to be proven, although it could be speculated that the latter is more likely due to the requirement of MT treatments to only be used below toxic threshold levels.

Current research in the field of CG microbial assemblage analysis focuses on the aerobic stability and quality of the fermentation rather than assigning any differences in community to MT degradation in CG. This molecular investigation characterises the promising result in the preceding chapter by identification of potential DON-degrading bacteria using DGGE/isolate sequencing whilst discussing the limitations of these techniques for CG silage.

4.3 Materials and methods

The molecular techniques employed in this chapter aim to elucidate which bacteria are possibly responsible for DON degradation in Chapter 3 and could be tested further as potential DON-degrading candidates in Chapter 5 (Figure 4.1).
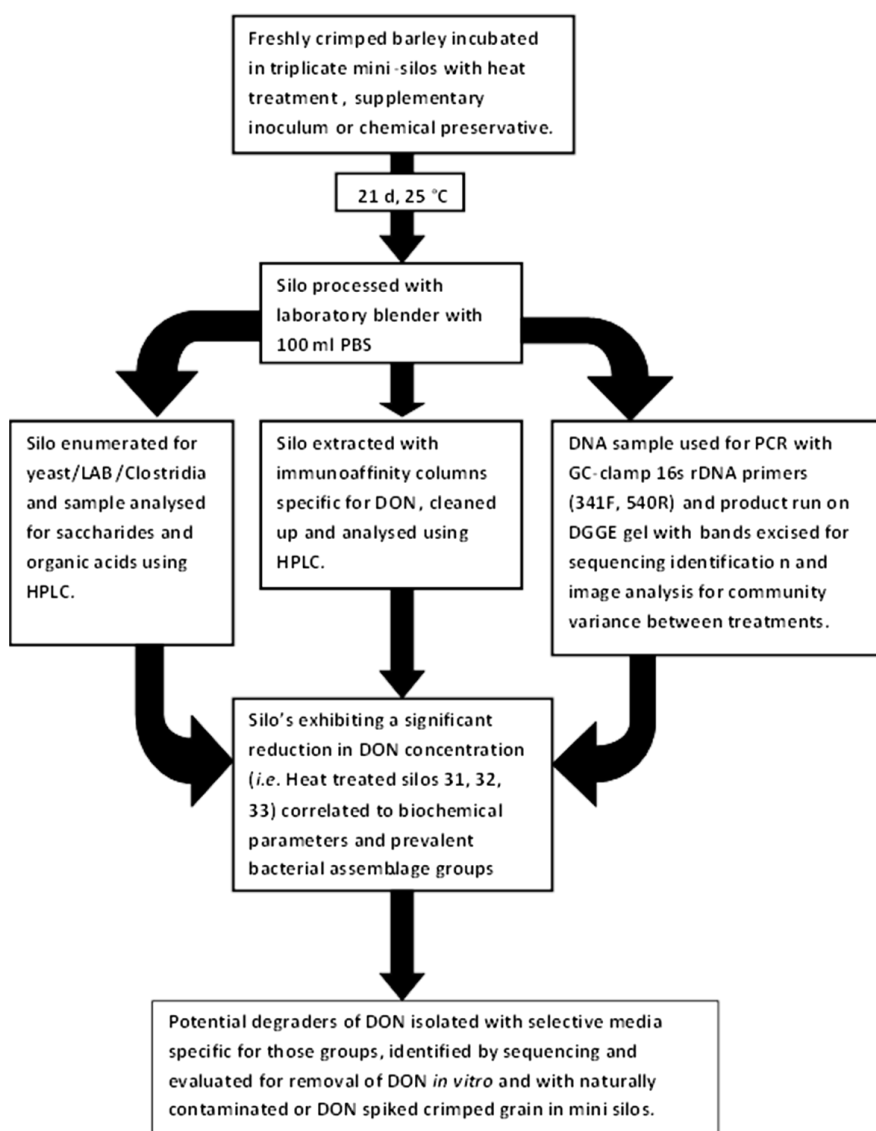
Figure 4.1. Schematic flow diagram highlighting the steps undertaken in chapters 3, 4 and 5.

4.3.1 DNA extraction of mixed community samples (CG silage).

DNA extraction of mixed community silo samples was achieved using a FastDNA™ spin kit

for soil (MP™ Biomedicals, USA) to avoid any skewing of community components that

may occur with phenol/chloroform-based extraction procedures which may be less

effective for gram-positives (Promega™ kit gave comparable results, Figure 4.2). The

manufacturer's protocol was followed with a few modifications. Briefly, 500 mg of re-

suspended CG silage (see section 3.3.4) was added to the Lysing matrix E tube followed by

978 µl sodium phosphate buffer and 122 µl M-T buffer. The rack of tubes was then

attached to a Vibrax platform and agitated at full speed for 2 min and the tubes centrifuged at 14000g for 10 min with the resulting supernatant added to a 2 ml microcentrifuge tube with 250 µl protein precipitation solution and mixed by inversion 10 times. The mixture was centrifuged and the supernatant added to 1 ml silica binding matrix solution in a 15 ml tube and re-suspended, allowing the solution to settle and removal of 500 µl to waste. The remaining liquid was used to re-suspend the matrix and 600 µl of the mixture was added to a SPIN™ filter tube and centrifuged at 14000g for 1 min and the eluent discarded. The pellet was re-suspended with SEWS-M solution, centrifuged (14000g, 3 min) to dry the matrix and SPIN™ filter and 50 µl of molecular grade water was added to the matrix and the sample centrifuged (14,000g, 1 min) into a fresh tube prior to quantification of DNA by Nanodrop™.

4.3.2 Extraction of DNA from isolated colonies

Confluent broth cultures (1 ml of tryptone soya broth, incubated anaerobically for 48 h at 30°C) were pelleted and rinsed twice with PBS. The pellet was then re-suspended in 500 µl of lysis solution (1 M sodium acetate, 200 mM tris-HCl, 500 mM NaCl, 10 mM EDTA, 1% SDS) and heated for 10 min at 95 °C in 1.5 ml microcentrifuge tubes before chilling on ice and adding 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1), inverting and incubating at 4 °C for 10 min. Samples were centrifuged at 10,000g for 6 min and 200 µl of supernatant removed to a fresh tube containing 100 µl sodium acetate (3 M, pH 5) and 1 ml isopropanol (100%, -20 °C), mixed and incubated on ice for 1 h. Samples were then centrifuged at 10,000g for 6 min and the supernatant removed leaving a pellet that was rinsed with 0.5 ml 70% ethanol and the pellet air dried by inverting each tube onto a sterile tissue in a laminar flow hood before being re-suspended in 30 µl low TE (10x

diluted TE buffer, pH 8, to limit enzymatic digestion), analysed using nanodrop for

purity/yield and diluted down to 30 ng/μl for PCR.

4.3.3 Polymerase chain reaction for DGGE and 16S rRNA sequencing

Primers based on those published by Muyzer and co-workers (1993) and were

synthesised by Eurofins Genomics GmbH (Ebersberg, Germany), reconstituted at 50

pMol/μl in molecular grade water and stored at -20 °C (Table 4.1). PCR reactions were set

up in thin walled dome-lid PCR tubes with 25 μl Taq 2x readymix (Bioline), 2 μl forward

primer, 2 μl reverse primer, 30 ng DNA template made up to 50 μl with PCR grade H_2O

and run on a Techne TC-512 PCR machine (Bibby Scientific, UK) using the touchdown

thermocycling program as follows: 5 min 94 °C, 1 min at 94 °C, 1 min 65 °C (decreasing

every 2nd cycle gradually to 55 °C (*I.e.*, decrease annealing from 65 > 55 °C over 20 cycles),

1 min 72 °C, then a further 10-15 cycles of this, 1 min at 94 °C, 1 min 55 °C, 1 min 72 °C

and a final extension at 72 °C for 7 min.

Table 4. 1. Primers used for PCR-DGGE and PCR-sequencing.

Primer specifics	Forward	Reverse
16S rRNA GC clamp for DGGE	341F (5'-CGCCCGCCGCGCGCGGCGGGCGGG GCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG-3')	540R (5'-ATTACCGCGGCTGCTGG-3')
16S rRNA for sequencing from DGGE bands	341F (5'-CCTACGGGAGGCAGCAG-3')	540R (5'-ATTACCGCGGCTGCTGG-3')
16S rRNA for sequencing from cultures	9F (5'-GAGTTTGATCCTGGCTCAG-3')	1512R (5'-CGGCTACCTTGTTACGACTT-3')

PCR (premixed with loading buffer) products were verified against a 100-1000BP ladder

(Bioline™ IV) on a 1% agarose gel in TAE buffer (40 mM Trizma-base, 1 mM EDTA-

disodium salt, pH 7.8 with acetic acid) pre-stained with SYBR safe (0.01%, Invitrogen™)

and run at 90 V for 40 min. Visualisation was achieved using an Applied Biosystems

GelDoc™ system and only single bands with insignificant smearing and low amounts of genomic template DNA were chosen for clean-up procedures/DGGE.

4.3.4 Denaturant gradient gel electrophoresis (DGGE)

DGGE involves the separation of duplex PCR products on a denaturant gel based on sequence G-C content. A G-C clamp is incorporated in the primer pair which is synthesised along with the template and acts as a hinge, preventing the sister strands completely separating as they migrate through denaturant and divide into sister strands. The G-C content regulates where on the gel denaturing occurs and migration dramatically slows forming a band, which can be used for banding pattern analysis and band identification by sequence PCR.

DGGE gels were run using a DCode™ Universal Mutation Detection System (Bio-rad, Hercules, USA). PCR products (20 µl + 2 µl + blue loading buffer) were pipetted into rinsed wells of a 0-30%, 30-60% or 45-70% denaturant gel submerged in tank of 7 L TAE buffer at 60 °C. Several gradients were trialled to find the optimum for fermented feed where bands could be spread over the whole length of the gel. 10% acrylamide gels were constructed with a Bio-rad gradient system attached to two syringes, each filled with the premixed denaturant solutions specific for each gradient made from 0 and 80% stock solutions (0%; 33.4 ml 30% acrylamide solution, 2 ml 50x TAE, made up to 100 ml with sterile H_2O, 80%; as 0% but with 32 ml formamide and 5.6 M urea). The glass gel plates were cleaned with acetone and one surface coated with rain repellent (Halfords, UK), clamped in place with PTFE spacers and sealed with high-vacuum grease (Dow Corning, USA). Prior to pouring the gel 100 µl of 10% Ammonium persulphate and 10 µl of TEMED (N,N,N-tetramethylethylenediamine) to each of the 25 ml denaturant mixes and aspirated

into two syringes attached to the gradient wheel. Carefully the wheel was rotated with a differential amount from each syringe expelled through a silicon tube fitted with a needle affixed to the top of the gel mould. When 1 cm from the top a 20 µl (18 well) comb was carefully inserted and the gel left to polymerise for 2 h before placing in the running tank, adding the PCR products mixed with loading buffer (4 ml glycerol, 6 ml 1x TE, 25 mg bromophenol blue, mixed 1:4 ratio with DNA) into each well and running at 64 V for 16 h at 60 ^{o}C. The gel was then separated from the glass plates and incubated for 30 min in the staining solution (TAE, 1 x SYBR green) on a shaking platform (IKA Vibrax VXR, 200 rpm) and visualised on a Gel-Doc system with UV trans-illumination (Gel-doc XR, Biorad). A sterile 1 ml filter tip was used to take a gel core from each distinctive band and inserted into a sterile 1.5 ml tube with 20 µl molecular grade water which was left for 12 h before using as PCR template. In addition, the DGGE gels were analysed with the Geldoc™ software using the presence or absence of bands to produce a binary matrix that was analysed in Minitab 16 using multivariate clustering of variables to produce a dendrogram.

4.3.5 PCR product clean-up, sequencing and BLAST searching

PCR clean up involves the removal of primers and PCR additives which will hinder the sequencing reaction and is achieved using Sureclean™ reagent (Bioline™, UK). Briefly, 20 µl of PCR reaction verified by electrophoresis was vortexed with 20 µl Sureclean in sterile 1.5 ml micro-centrifuge tubes and incubated for 10 min at room temperature and centrifuged at 14000g for 20 min. The supernatant was removed by pipette and the pellet re-suspended in 70% ethanol by vortexing for 10 s followed by another 20 min centrifugation and the tube upturned onto a piece of sterile Kimwipe until all liquid has evaporated (in laminar-flow hood). Another 70% EtOH wash, centrifugation and

evaporation using a fresh Kimwipe® was followed by re-suspending in 20 µl molecular grade water and the concentration of DNA measured using a Nanodrop™ spectrophotometer and diluted to 1.5 ng/µl of which 15 µl was sent away for Sanger sequencing with 10 pmol forward primer only (Eurofins Genomics, Germany). Chromatogram data was handled with Mega 5.1 (Tamura *et al.*, 2011.) where sequence quality was evaluated before uploading to an online sequence alignment database (http://blast.ncbi.nlm.nih.gov) for a BLASTN nucleotide search of similar sequences. The alignment with the highest identities (%) is listed for each bacteria type with the accession number. Only typed full gene sequences were used for alignment.

4.3.6 Isolation of DON biotransformation candidates in silo samples with selective media

Following analysis by DGGE the original silo extracts were 10x serially diluted with PBS and 20 µl plated onto selective agar following the ingredients listed in The Handbook of Microbiological Media for the Examination of Food (Atlas, 2006). Media included: *Bacillus* selective agar (starch, polymixin B): *Bacillus* spp.; McKonkey agar: *Escherichia* spp.; Slanetz and Bartley agar: gram negative Enterrococci; *Bacillus cereus* agar: *Bacillus* spp. and SODLAC agar (sodium lactate); lactate metabolising propionic-type bacteria or lactate metabolising LAB. In addition, SODLAC broth was employed by serial enrichment with 100 ml inoculated with 100 µl of silo extract, incubated anaerobically for 48 h at 25 °C and 100 µl used to inoculated another 100 ml for 48 h, and repeat, followed by plating on MRS (lactic acid bacteria) and SODLAC agar (lactate metabolising bacteria). The use of SODLAC medium stemmed from the metabolites measured in the heat treated silos, which included propionic acid. All isolates were cultured in Tryptone soya broth (25 °C, anaerobically), snap frozen with 15% glycerol and prepared for PCR.

4.4 Results

4.4.1 Comparison of Promega™ and MO™ soil kits for DNA extraction

The similarity in terms of banding pattern of the two different DNA extraction kits (Figure 4.2), for example 31 and 31A, suggests that the two different methods of DNA extraction yield almost identical community profile and are not excluding any key assemblages due to varying membrane tenacity between groups (*i.e.* Gram positive/negative). The only slight differences in banding patterns, only observable with magnified image analysis and illustrated with a dendrogram of similarity (Figure 4.6), highlight the importance of universal PCR primer design, PCR reaction optimisation and accurate DNA concentration measurements designed to eliminate bias in the final gel. Since the differences are not significant for the purposes of directing the bacteria isolation strategy, the Promega™ or MO™ kits were considered interchangeable, and the MO™ soil kit was used for the remaining extractions. One issue of a potential key bacteria not being represented on the gel is discussed further in 4.5.4.
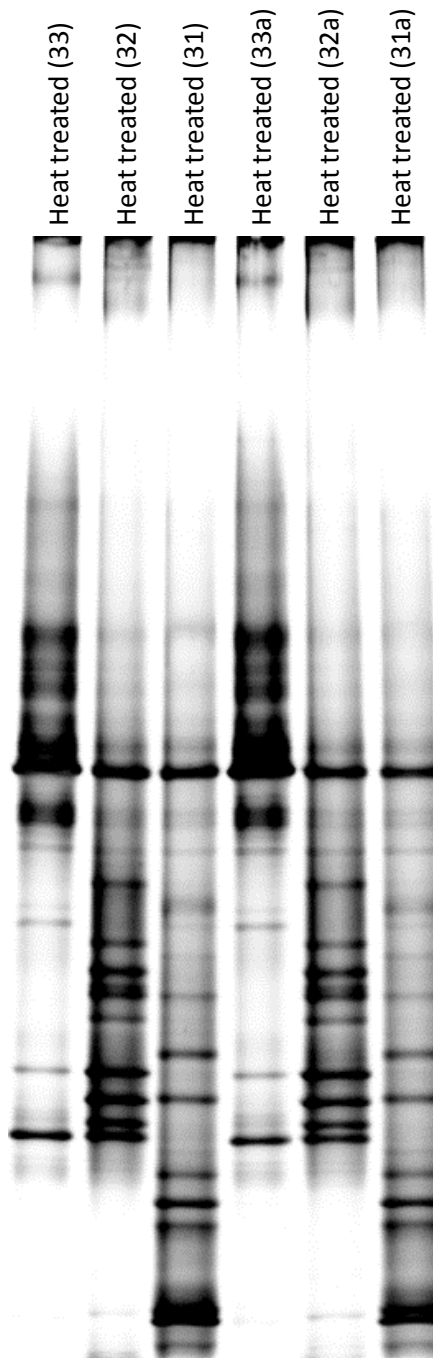
Figure 4.2. A comparison of the DGGE profiles produced with either MO soil or Promega cellular DNA extraction kits to check whether different kits would have a bearing on the DNA extraction efficiency and possible skew of bacterial community components. The silo number is given with the treatment, with "a" denoting the Promega extraction.

4.4.2 Bacterial assemblage differences between treatments following treatment of silos with different biological and chemical additives
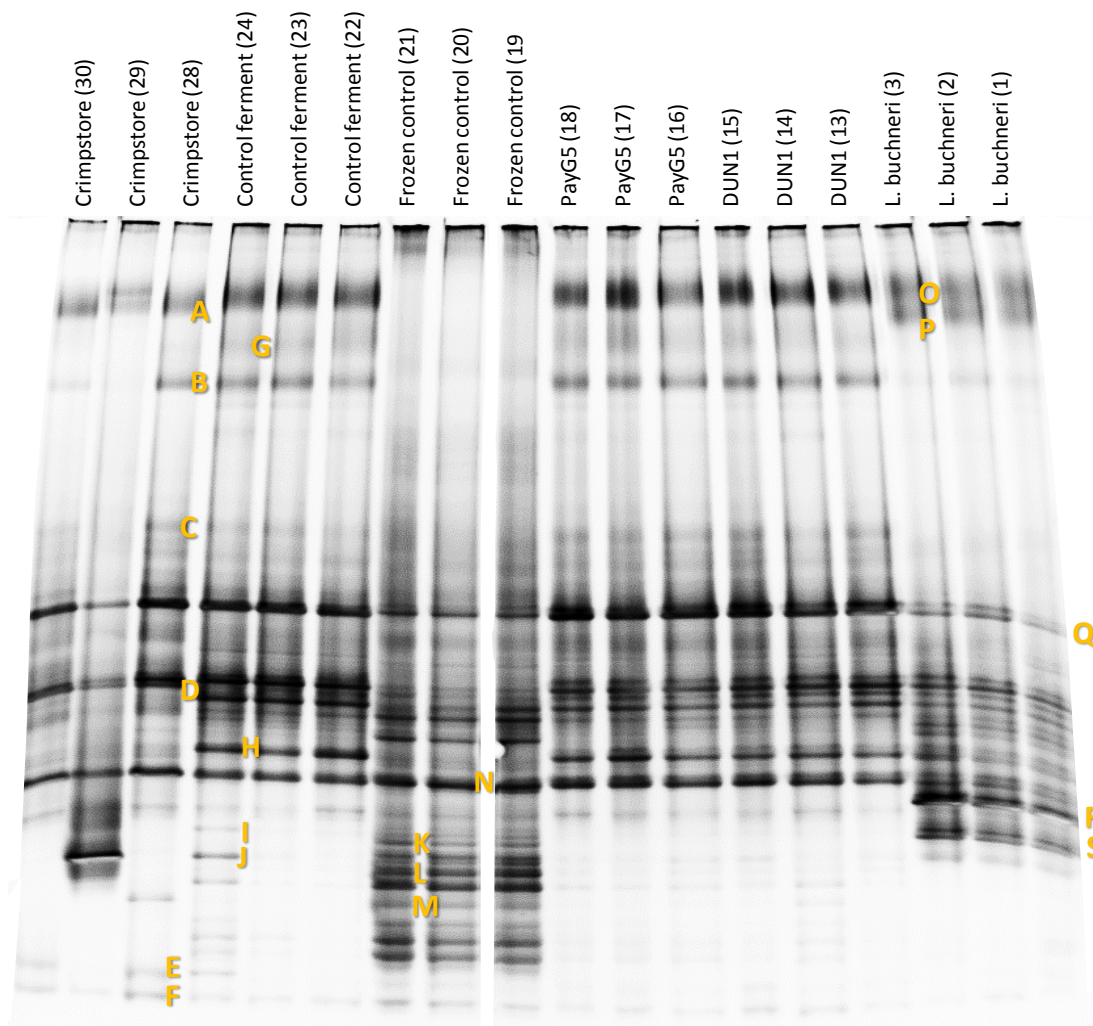
Figure 4.3. DGGE gel image comparing triplicate silo treatments (numbered) with sequenced bands annotated with a letter that corresponds to an aligned sequence and bacterial species in Table 4.2.

When identically migrating bands between lanes were excised and sequenced there was excellent sequence similarity, which along with the ability to sequence and identify all bands attempted illustrates the suitability of this screening technique for CG assemblage analysis. The dendrograms (Figure 4.4, 4.6) illustrate how the presence or absence of DGGE bands can be used to derive differences in community make-up between silo treatments. It can be noted that a single bacterial strain may produce more than one band on the gel (DGGE of isolate DNA not shown).

Concerning the use of additives for influencing the bacterial assemblage in each silo, it is apparent that there is a significant difference between the frozen control and all the treatments (Figure 4.3, 4.4). The frozen material in general has a less distinct banding consisting of epiphytic bacteria such as *Pantoea agglomerans* (formerly: *Enterobacter agglomerans*), *Erwinia* sp. and *Escherichia hermannii* with those LAB instrumental in fermentation not represented significantly at this stage suggesting the level of inoculum present initially is not indicative of 21 d fermentative assemblage.

L. buchneri produced the most significant shift in final community assemblage of the LAB treatments, with only hetero and homofermenters significantly represented, the majority of which was *L. buchneri* (Figure 4.3). Furthermore, the consistency of banding between the three silos is indicative of *L. buchneri* dominating the fermentation, reducing the pH and effectively out-competing the other groups. Conversely, the addition of the *L. plantarum* strains DUN1 and PayG5 did not strictly influence natural succession in this case as *L. plantarum* was also present in the control fermentation, yet they were found as distinct clusters according to banding pattern and fermentation characteristics were more effective. Crimpstore® was in an intermediate cluster between the frozen starting material and the control fermentation proving how chemical preserved CG still possesses the ability to support a natural fermentation profile, albeit at a slower rate and with a delayed onset.

Table 4. 2. BLAST search identities and aligned likely bacteria species for excised bands in Figure 4.3. Many of the bands are in common with many of the different treatments and others are unique to that treatment. Asterisks indicate sequence relates to plant chloroplasts/mitochondria (evolutionary artefacts).

Band code	Closest relative (accession number)	Identities (%)
A	*Lactobacillus paralimentarius* (JN863628.1)	148/151(98%)
B	*Lactobacillus plantarum* (AB572043.1)	31/32 (97%)
C	*Bacillus sp.* (HM598361.1)	31/31 (100%)
D	*Lactobacillus paracasei* (GU424995.1)	52/56 (93%) & 26/28 (93%)
E, F*	*Triticum aestivum* (wheat mitochondrion) (GU985444.1)	111/111 (100%)
G	*Lactobacillus plantarum* (JQ712984.1)	131/141(93%)
H	*Lactobacillus sp.* (AB794060.1)	132/146 (90%)
I	*Enterobacter sp.* (CP000653.1)	137/146 (94%)
J	*Pantoea agglomerans* (AB681812.1)	147/149 (99%)
K	*Pantoea eucalypti* (EF688009.1)	136/145 (94%)
L	Enterobacteriaceae bacterium (JX067700.1)	133/140 (95%)
M	*Erwinia sp.*(GQ405203.1)	92/98 (94%) & 17/17 (100%)
N*	*Hordeum vulgare* subsp. *vulgare* chloroplast (EF115541.1)	115/125 (92%)
O	*Lactobacillus buchneri* (JQ249065.1)	149/156 (96%)
P	*Lactobacillus buchneri* (JQ612702.1)	147/150 (98%)
Q	*Lactobacillus plantarum* (EF439684.1)	134/144(93%)
R	*Lactobacillus kefiri* (AB362680.1)	145/154(94%)
S	*Lactobacillus buchneri* (JQ249047.1)	146/148(99%)

Bacterial community correlation

| 100.00 | 87.32 | 74.63 | 61.95 |

Crimpstore (30)
Crimpstore (29)
Crimpstore (28)
Control ferment (24)
Control ferment (23)
Control ferment (22)
PayG5 (18)
DUN1 (15)
DUN1 (14)
DUN1 (13)
PayG5 (17)
PayG5 (16)
L. buchneri (3)
L. buchneri (2)
L. buchneri (1)
Frozen control (21)
Frozen control (20)
Frozen control (19)
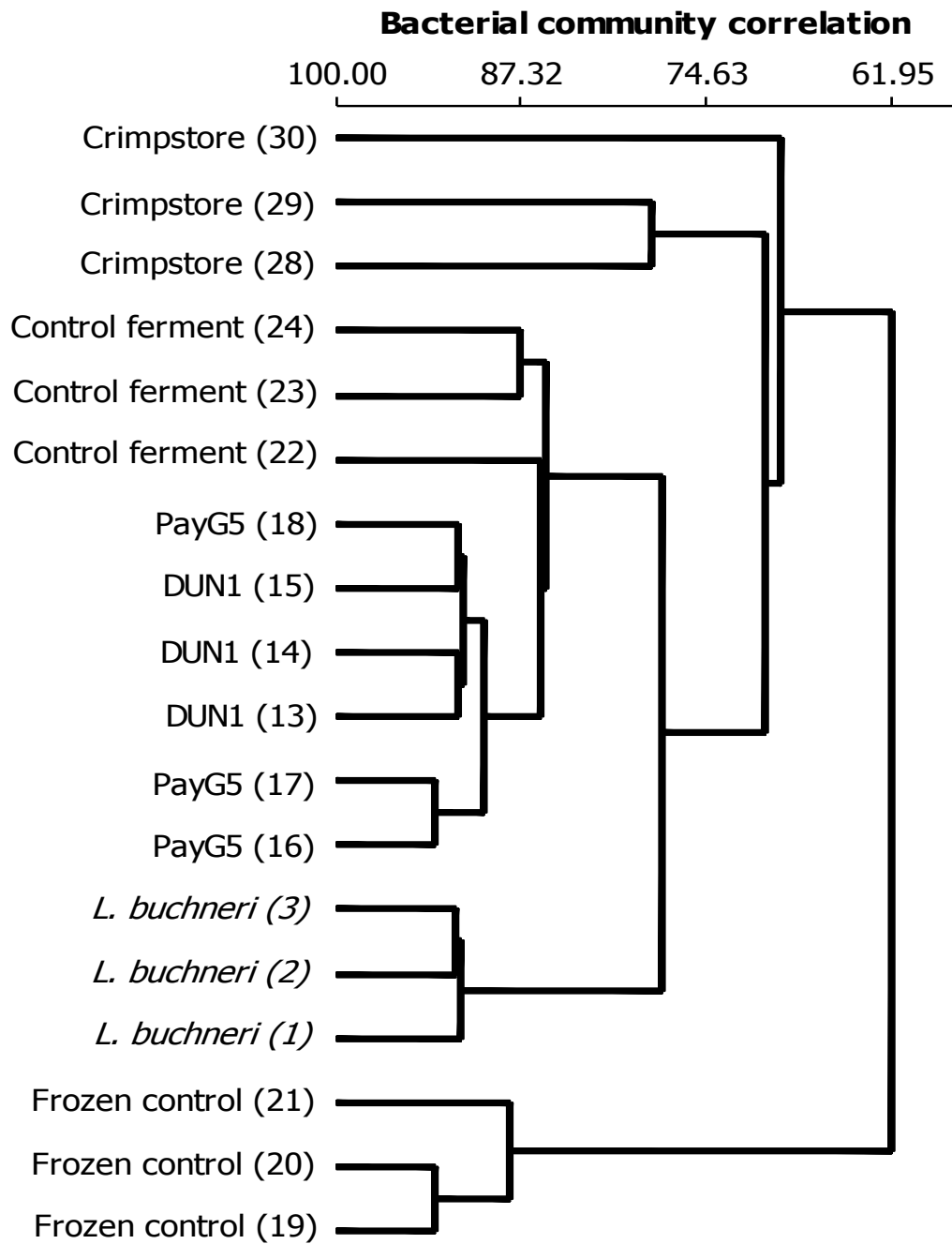
Figure 4.4 Dendrogram illustrating the clustering of silo treatments in terms of 16S rRNA banding pattern following DGGE. The position of the joiners is indicative of correlational distance (%).

4.4.3 Identity of dominant bacteria within the heat treated silos where DON levels were reduced

Analysis of the heat treated silos (31, 32, and 33) which had reduced recovery of DON

(58.9, 24.1, and 10.7% of initial respectively) indicates a far greater diversity between

silos than the other treatments in terms of bacterial taxonomy and banding pattern differences (Figure 4.5, 4.6).
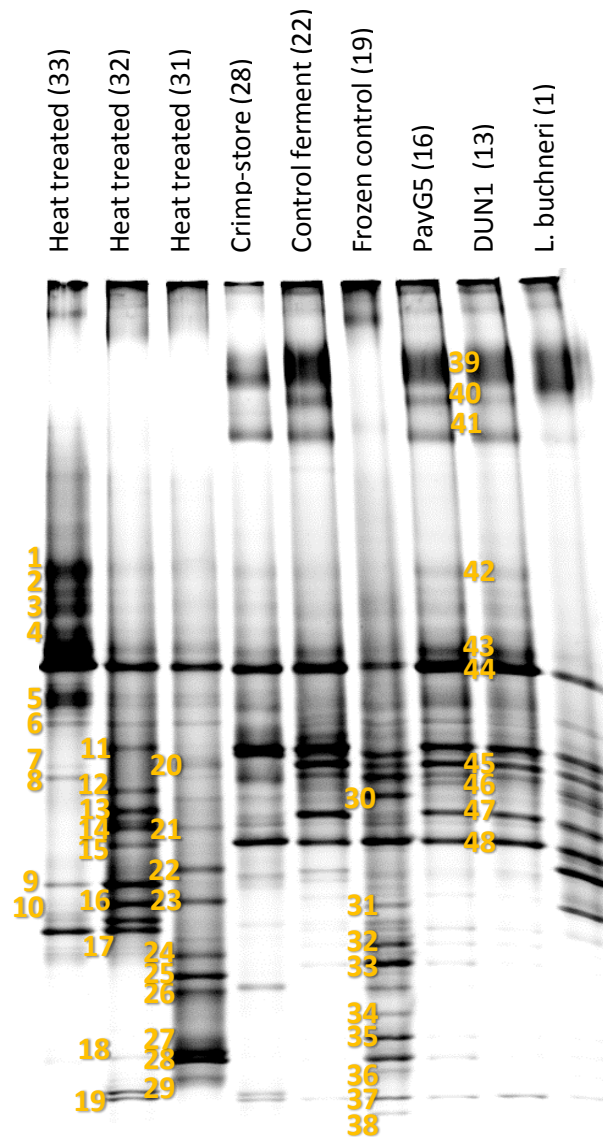
Figure 4.5. DGGE gel image to compare heat treated silos (31, 32, 33) with the other treatments with sequenced bands annotated with a letter that corresponds to an aligned sequence in BLAST in Table 4.3.

Table 4. 3. BLAST search identities and aligned likely bacteria species for excised DGGE bands in Figure 4.5. Many of the bands are in common with many of the different treatments and others are unique to that treatment. All DGGE bands able to PCR and sequence for heat treatment silos 31, 32 and 33 are shown.

Band code	Closest relative (accession number)	Identities (%)
	Heat treated (33)	
1	*Weissella cibaria* (JN851741.1)	138/147 (94%)
2	*Weissella cibaria* (JN851741.1)	146/149 (98%)

3	*Weissella sp*. (EF468052.1)	132/138 (96%)
4	*Weissella cibaria* (JN851741.1)	149/150 (99%)
5	*Weissella cibaria* (JN851741.1)	138/148 (93%)
6	Delta proteobacterium (AY162123.1|)	36/37 (97%)
7	*Bacillus coagulans* (JQ073764.1)	107/136(79%)
8	*Vibrio sp.* (DQ479374.1)	40/42 (95%) & 33/36 (92%)
9	*Yersinia massiliensis* (JX429059.1)	130/150 (87%)
10	*Pantoea punctata* (HQ018863.1)	119/147 (81%)
	Heat treated (32)	
11	*Bacillus licheniformis* (JN000303.1)	102/111 (92%)
12	*Bacillus licheniformis* (JN540805.1)	113/133 (85%)
13	*Bacillus licheniformis* (JX912559.1)	118/129 (91%)
14	*Bacillus sp.* (FM865970.1)	130/136 (96%)
15	*Bacillus subtilis* (HQ425655.2)	140/151 (93%)
16	*Paenibacillus sp.* (AJ582394.1)	52/55 (95%)
17	*Micrococcaceae bacterium* (AY177727.1)	87/101 (86%)
18	*Bacillus sp.* (AY433824.1)	50/54 (93%)
19	*Microbacterium aerolatum* (FJ944691.1)	109/128 (85%)
	Heat treated (31)	
20	*Klebsiella pneumoniae* (AF130981.1)	130/149 (87%)
21	*Enterobacter agglomerans* (AF130886.2)	131/151 (87%)
22	*Pantoea agglomerans* (HM161853.1)	127/150 (85%)
23	*Enterobacter agglomerans* (AF130886.2)	128/149 (86%)
24	*Klebsiella pneumoniae* (AB642256.1)	118/135 (87%)
25	*Enterobacter agglomerans* (AF130886.2)	118/135 (87%)
26	*Enterobacter agglomerans* (AF130886.2)	137/149 (92%)
27	*Escherichia hermannii* (JX968501.1)	151/152 (99%)
28	*Enterobacter agglomerans* (AF130886.2)	149/151 (99%)
29	*Escherichia hermannii* (JX968501.1)	148/150 (99%)
	Frozen control (19)	
30	*Pantoea agglomerans* (HM224395.1)	96/113 (85%)
31	*Enterobacter sp.* (FJ355965.1)	131/145 (90%)
32	Enterobacteriaceae bacterium (JX067700.1)	143/146 (98%)
33	*Pantoea agglomerans (EU849113.1)*	138/143 (97%)
34	*Pantoea agglomerans* (EU849113.1)	139/144 (97%)
35	*Pantoea agglomerans* (KC178591.1)	140/143 (98%)
36	*Escherichia hermannii* (JX968501.1)	142/146 (97%)
37	*Pantoea agglomerans* (DQ065752.1)	123/133 (92%)
38	*Triticum aestivum* (wheat mitochondrion) (GU985444.1)	99/100 (99%)
	DUN1 (13)	
39	*Lactobacillus sakei* (JN851763.1)	143/146 (98%)
40	*Lactobacillus plantarum* (JN863682.1)	142/147 (97%)
41	*Lactobacillus plantarum* (JN859533.1)	141/147 (96%)
42	*Lactobacillus plantarum* (JN863682.1)	137/142 (96%)
43	*Lactobacillus plantarum* (EF439684.1)	132/141 (94%)
44	*Lactobacillus plantarum* (EF439684.1)	135/145 (93%)
45	*Lactobacillus brevis* (JN863690.1)	117/142 (82%)
46	*Pediococcus ethanolidurans* (AB706354.1)	103/137 (75%)
47	*Lactobacillus crustorum* (KC571196.1)	127/141 (90%)
48	*Hordeum vulgare subsp. vulgare* (barley chloroplast) (EF115541.1)	113/118 (96%)
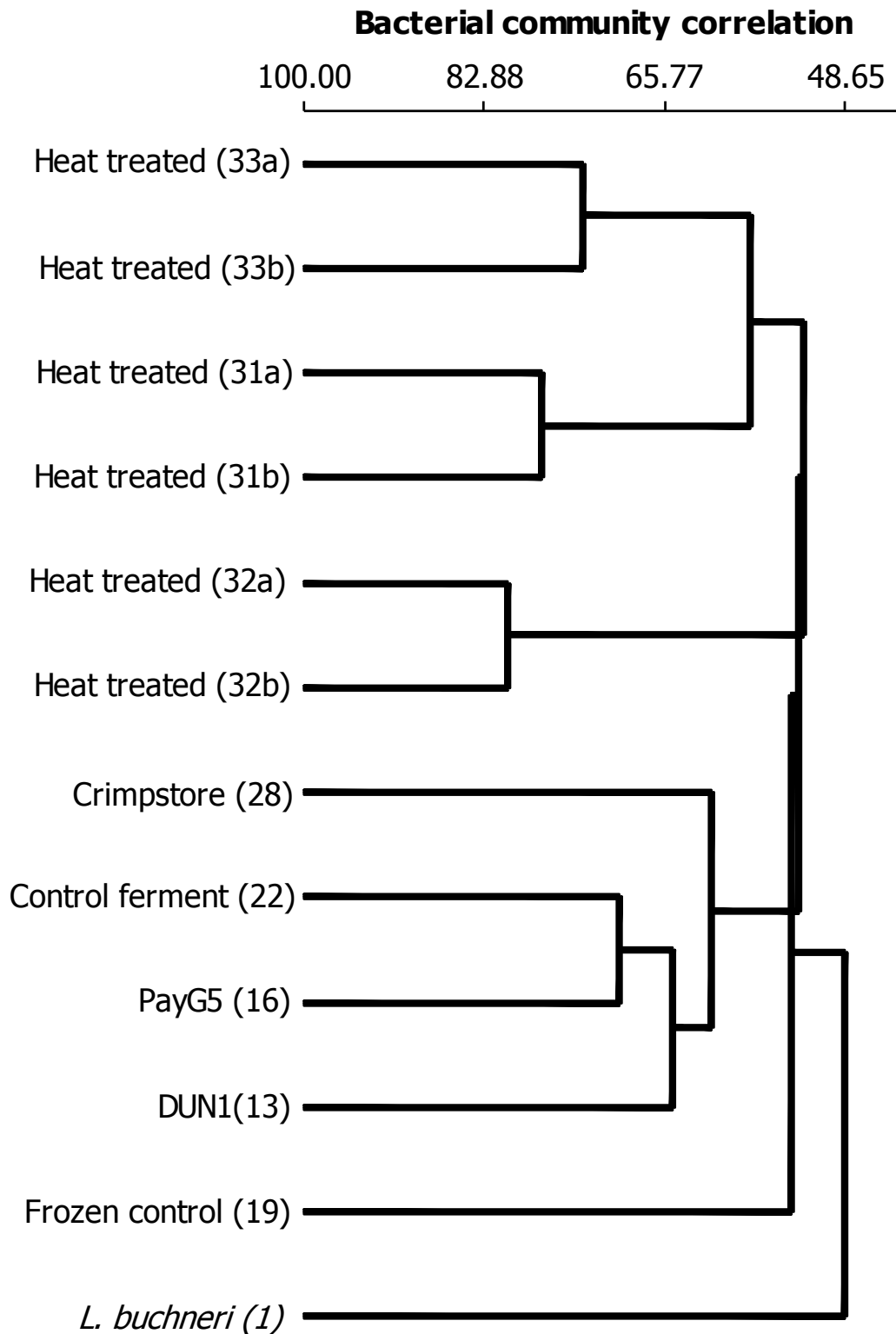
Figure 4.6. The cluster analysis undertaken of the binary tabulated band analysis data is illustrated as a centroid dendrogram with treatments linked by correlation coefficient (%). Silo treatment is shown with the silo number.

Silo 31, the heat treated silo with the least DON degradation, was generally colonised with *Pantoea agglomerans, E. hermannii and Klebsiella pneumoniae*. These are species more often found in early stage grass silage or late stage high moisture content silage (Li and Nishino, 2013). Silo 32 (24.1% DON recovery) was found to be populated by *Bacillus* sp. such as *B. subtilis* and *B. licheniformis* in addition to lesser prominent groups including Micrococcaceae, Microbacteriacea and *Paenibacillus* sp. These species are usually found in aerobically deteriorated silages and are categorised as highly heat resistant spore forming silage bacteria by Driehuis (2013). Silo 33 which had the least recovery of DON (10.7%) was predominantly colonised with *Weissella cibaria,* a lactic acid bacteria in the Leuconostocaceae family more often evident in maize silage (Pang *et al.*, 2011), early grass silage fermentation, non-spore forming, aerotolerant, heterofermentative and generally regarded as safe for consumption (Björkroth *et al.*, 2002). None of the heat treated silos produced any viable yeast counts, therefore only the bacterial assemblage analysis was deemed worthwhile.

4.5.4 Selective and enrichment media derived isolates

Once the bacterial community of each of the heat treated silos was determined the suite of selective agar was employed to isolate vegetative cells from morphologically distinct colonies. Whilst some could be grown directly following dilution of silo extract, others required a period of enrichment in SODLAC broth to screen for propionic acid producing bacteria and other lactic acid catabolising bacteria. *Hafnia alvei* and *Leuconostoc mesenteroides* colonies were only found to be present using this technique as they did not appear in the DGGE analysis.

Table 4. 4. Selective agar employed to isolate possible degraders of DON as recorded in the DGGE analysis. Specificities as follows: *Bacillus* selective agar (starch, polymixin B): *Bacillus* sp.; MacKonkey agar: *Escherichia* sp.; Slanetz and Bartley agar: gram negative Enterrococci; *Bacillus cereus* agar: *Bacillus* sp. and SODLAC agar/broth (sodium lactate); lactate metabolising propionic bacteria. *Anaerobic.

Selective conditions	Isolate code	Closest relative (accession number)	Identities (%)
Silo: 31 *Bacillus* selective agar*	WW1	*Leuconostoc mesenteroides* subsp. *mesenteroides* (GU470933.1)	540/543 (99%)
Silo: 31 McKonkey agar*	WW2	*Pantoea agglomerans* (HE647624.1)	528/528 (100%)
Silo: 31 McKonkey agar*	WW3	*Leuconostoc mesenteroides* (JN851765.1)	525/525 (100%)
Silo: 33 Slanetz and Bartley*	WW5	*Leuconostoc mesenteroides* subsp. *mesenteroides* (JN863609.1)	526/526 (100%)
Silo: 33 *Bacillus* selective*	WW6	*Leuconostoc mesenteroides* subsp. *mesenteroides* (JN863609.1)	526/526 (100%)
Silo: 32 *Bacillus* selective*	WW7	*Bacillus licheniformis* (HM753626.1)	501/522 (96%)
Silo: 32 Slanetz and Bartley*	WW8	*Leuconostoc mesenteroides* (JN851765.1)	522/522 (100%)
Silo: 33 *Bacillus* selective*	WW9	*Leuconostoc mesenteroides* (JN851765.1)	524/524 (100%)
Silo: 32 *Bacillus* selective*	WW10	*Bacillus subtilis* (HQ425655.2)	527/527 (100%)
Silo: 32 *Bacillus* selective*	WW11	*Leuconostoc mesenteroides* (JN851765.1)	525/525 (100%)
Silo: 31 Slanetz and Bartley*	WW12	*Leuconostoc mesenteroides* subsp. *mesenteroides* (KC213923.1)	527/527 (100%)
Silo: 31 MacKonkey agar*	WW13	*Pantoea agglomerans* (HE647624.1)	526/526 (100%)
Silo: 31 *Bacillus cereus* agar*	WW14	*Pantoea agglomerans* (HE647624.1)	525/525 (100%)
PM33ANB SODLAC enrichment broth to MRS agar*	WW15	*Leuconostoc mesenteroides* subsp. *mesenteroides* (KC213923.1)	530/530 (100%)
P33aii SODLAC broth to MRS agar*	WW16	*Hafnia alvei* (JX480505.1)	527/527 (100%)
33b SODLAC broth to MRS agar*	WW17	*Leuconostoc mesenteroides* (KC213923.1)	525/525 (100%)
31 SODLAC broth to MRS agar*	WW18	*Hafnia alvei* (JX480505.1)	448/452 (99%)
P33a SODLAC broth to MRS agar*	WW19	*Hafnia alvei* (JX480505.1)	518/518 (100%)
PM33C SODLAC broth to MRS agar*	WW20	*Leuconostoc mesenteroides* (JN851765.1)	533/533 (100%)
P33A SODLAC broth to SODLAC agar*	WW21	*Lactobacillus plantarum* (JN863682.1)	525/525 (100%)
P33B SODLAC broth to MRS agar*	WW22	*Leuconostoc mesenteroides* (JN851765.1)	525/525 (100%)
33A SODLAC broth to MRS agar	WW23	*Leuconostoc mesenteroides* (JN851765.1)	525/525 (100%)
P32Anc SODLAC broth to SODLAC agar*	WW24	*Bacillus sp.* (GU939695.1)	414/535 (77%)
P32Anc SODLAC broth to MRS agar	WW25	*Leuconostoc mesenteroides* (KC213923.1)	515/515 (100%)
P32a SODLAC broth to SODLAC agar*	WW26	*Hafnia alvei* (JX480505.1)	531/532 (99%)
P32a SODLAC broth to MRS agar*	WW27	*Hafnia alvei* (JX480505.1)	529/529 (99%)

Table 4.5. Bacterial strains isolated from silos 31-33 using SODLAC agar and broth under anaerobic conditions (25 °C).

Isolate code	Closest relative (accession number)	Identities (%)
33A	*Weissella cibaria* (AB761300.1)	841/848 (99%)
P33BM	*Leuconostoc mesenteroides subsp. dextranicum* (AB795649.1)	831/831 (100%)
33D	*Weissella cibaria* (AB761300.1)	857/861 (99%)
M8 PM33ANA	*Weissella cibaria* (HF562960.1)	825/825 (100%)
M10 PM33ANB	*Leuconostoc mesenteroides* (KC545921.1)	799/799 (100%)
M11 PM33AM	*Leuconostoc mesenteroides* (KC545921.1)	730/730 (100%)
M12 33C	*Weissella cibaria* (AB593347.1)	829/834 (99%)
M14 P33AM	*Hafnia alvei* (JX480505.1)	830/830 (100%)
M15 33B	*Leuconostoc mesenteroides* (HM218680.1)	838/840 (99%)
M16 PM32ANC	*Leuconostoc mesenteroides* (JQ680419.1)	843/844 (99%)
M17 PM32D	*Leuconostoc mesenteroides* (KC545921.1)	842/843 (99%)
M18 PM32BM	*Leuconostoc mesenteroides* (KC545921.1)	842/843 (99%)
M19 P32AM	*Hafnia alvei* (JX480505.1)	822/823 (99%)
M20 P31B(2)M	*Leuconostoc mesenteroides* (JQ680419.1)	829/830 (99%)
M21 P31BM	*Leuconostoc mesenteroides subsp. dextranicum* (AB795649.1)	828/828 (100%)
M22 PMAN31CM	*Leuconostoc mesenteroides* (JQ680419.1)	823/824 (99%)
M23 PM31AM	*Leuconostoc mesenteroides* (JQ680419.1)	819/822 (99%)

Following the apparent abundance of *Leu. mesenteroides* in the selective media and SODLAC enrichment experiments (also *H. alvei* to a lesser extent) but the conspicuous absence in the DGGE gels it was necessary to check that the universal primers prescribed by Muyzer (1993) and used in the current PCR-DGGE have specificity required for the 16s rRNA sequence in *Leu. mesenteroides* and *H. alvei*. By nesting the DGGE primers (labelled yellow, Figure 4.7) within the partial 16s rRNA gene sequence obtained from the isolates in question it was evident that there was a primer-sequence mismatch for *Leu. mesenteroides* (labelled red), but not *H. alvei* or *W. cibaria* (positive DGGE bands). This would perhaps explain why this PCR fragment did not appear in the DGGE gel, and since it could be a major component of the bacterial assemblage in the heat treated silos it does illustrate a flaw in the use of these much employed universal primers. Further research confirmed that this A→T base change is known to be an identifying characteristic of the Leuconostocaceae (Walter *et al.*, 2001). The same author suggests that only 90-99% of the most numerous species in the community are effectively detected using the universal primer technique, therefore to monitor the minority members of the assemblage, more selective primers need to be designed based on pilots culture dependant identification

experiments. However, *in silico* and environmental trialling of a range of universal primers by Klindworth *et. al.* (2013) for classical and next generation sequencing found the 341F-540R primer pair (used in the current work) to have the best coverage for bacteria overall, although a larger fragment of 464bp could be used with only a slight reduction in species coverage (341F-805R).

M4; *Weissella cibaria* gene for 16S ribosomal RNA, partial sequence, strain: qz140 Sequence ID: dbj|AB761300.1|

```
301 GATCGGCCAC AATGGGACTG AGACACGGCC CATACTCCTA CGGGAGGCAG CAGTAGGGAA
361 TCTTCCACAA TGGGCGAAAG CCTGATGGAG CAACGCCGCG TGTGTGATGA AGGGTTTCGG
421 CTCGTAAAAC ACTGTTGTAA GAGAAGAATG ACATTGAGAG TAACTGTTCA ATGTGTGACG
481 GTATCTTACC AGAAAGGAAC GGCTAAATAC GTGCCAGCAG CCGCGGTAAT ACGTATGTTC
```

M14; *Hafnia alvei* strain 26 16S ribosomal RNA gene, partial sequence
Sequence ID: gb|JX480505.1|

```
241 AGAGGATGAC CAGCCACACT GGAACTGAGA CACGGTCCAG ACTCCTACGG GAGGCAGCAG
301 TGGGGAATAT TGCACAATGG GCGCAAGCCT GATGCAGCCA TGCCGCGTGT ATGAAGAAGG
361 CCTTCGGGTT GTAAAGTACT TTCAGCGAGG AGGAAGGCAT TGTGGTTAAT AACCGCAGTG
421 ATTGACGTTA CTCGCAGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACG
```

M15; *Leuconostoc mesenteroides subsp. mesenteroides* strain NM168-5 16S ribosomal RNA gene, partial sequence Sequence ID: gb|HM218680.1|

```
301 CACGGCCCAA ACTCCTACGG GAGGCTGCAG TAGGGAATCT TCCACAATGG GCGAAAGCCT
361 GATGGAGCAA CGCCGCGTGT GTGATGAAGG CTTTCGGGTC GTAAAGCACT GTTGTATGGG
421 AAGAACAGCT AGAATAGGAA ATGATTTTAG TTTGACGGTA CCATACCAGA AAGGGACGGC
481 TAAATACGTG CCAGCAGCCG CGGTAATACG TATGTCCCGA GCGTTATCCG GATTTATTGG
```

Figure 4.7. Partial 16s rRNA sequences for 3 representative heat-treated silo bacteria, with overlayed universal p1, p2 primers highlighted in yellow and base mismatches highlighted in red

4.5 Discussion

In general, the PCR-DGGE method of assemblage characterisation has proven to be an invaluable screening technique concerning the determination of potential DON degrading bacteria in CG feed as there was a high level of concordance between banding patterns of the same sample on different gels and almost every excised band yielded good sequence data facilitating reliable bacteria identification that could be confirmed when an isolate was obtained and sequenced. Whilst the PCR-DGGE-SEQ data gave sequence similarities below the 99% required for authentication, once the corresponding isolate was sequenced again with a larger fragment the identification was more robust.

Bacterial assemblage analysis of CG silos using PCR-DGGE-SEQ was shown to yield robust and reproducible data for all samples leading to directed isolation of viable relevant bacteria for further investigation in Chapter 5. The validity of the technique for fermented CG was confirmed following sequencing of colonies in the isolation phase which corresponded well to the genomic content of the silos. The retrospective lack of primer specificity for *Leu. mesenteroides*, a LAB instrumental in the early fermentation phase was addressed by employing additional culture dependant methods, although it would be prudent for future studies to undertake a pilot colony sequencing experiment to verify the universal primers prescribed by Muyzer *et al.* (1993) give sufficient species coverage without significant primer mismatch. Whilst a 1bp mismatch may not reduce the efficacy of PCR for single colonies, the competitiveness nature of the DGGE PCR reaction and secondary structure of 16S rRNA may dictate whether a certain product appears on the gel (Klindworth *et al.*, 2013, Neilson *et al.*, 2013). The use of both culture-dependant and independent techniques can complement each other, although care needs to be taken in interpretation. For example, not all species form CFU under laboratory conditions, even if a range of media and incubation conditions are provided. DGGE on the other hand yields comprehensive semi-quantitative species information in that band intensity can be arbitrarily related to copy number, although bacterial viability is not confirmed, and the influence of barley DNA content during the PCR template normalisation could bias the reaction by competing with the bacterial DNA for the initial PCR cycles. Moreover, preferential amplification of certain fragments due to the secondary structure of the 16S rDNA template, which is hypothesised by Neilson and colleagues as the main cause of multiple DGGE bands harbouring identical sequences, could be a significant method artefact when considering community diversity analysis

(Neilson *et al.*, 2013). However, the highly reproducible community structure profiles that can be obtained with DGGE provide excellent snapshots of functional diversity and more generalised shifts in community structure, and DGGE therefore an excellent tool when comparing bulk changes in large numbers of samples.

Alternatively, the use of clone library techniques where metagenome DNA/RNA is inserted into recombinant *Escherichia coli* for generation of clonal fragments, has often been viewed as preferential to DGGE as reads can be 5 times the length leading to more reliable identification in BLAST and sequences can be obtained from species not exhibited as a band on the DGGE gel. It can also be complementary to DGGE as clonal fragments can then be run as nested-PCR products concurrently on a DGGE gel for definitive identification of community bands based on migration distance, whilst maximising gene coverage (Handschur *et al.*, 2005).

Recent work has explored the possibility of carrying out DGGE on alternative functional genes to 16S rRNA, including those involved in primary metabolism; therefore providing phylogenetic and metabolic diversity information within the same DGGE experiment (Hallin *et al.*, 2006). Moreover, the use of non-universal primers enables low abundance groups that would not usually yield visible bands to be adequately amplified/identified. Taking this further, it may be possible to PCR homofermentative and heterofermentative separately or even screen for de-epoxidase/cytochrome p450 genes/RNA within the silo and selectively identify/isolate candidate DON-metabolising bacteria, specifically those transcribing candidate genes. Such work is only feasible if the corresponding gene is conserved and available in a searchable online database such as BLAST (NCBI) or the sequence of a particular DON-degrading enzyme/protein is published enabling

degenerate primers designed according to codon sequence to amplify homologous DNA or RNA sequences across all microbial taxa with PCR or RT-PCR respectively (Hakki and Akkaya, 2001). With the rapid development of next generation sequencing it is also possible to derive species and metabolic diversity by extracting the metagenome, translating the DNA/RNA *in silico* to functional protein structures, which are then linked *post hoc* to taxa with already annotated genomes (Mardis, 2008).

When considering the current suite of results it is apparent that the initial epiphytic community can only predict the integrity and community richness of naturally inoculated silage in the early stages of fermentation, as after that it is evident that even if LAB are below the limit of detection in DGGE at time zero, they can still go forth to dominate the mature silage with bands representing early epiphytic species (*i. e., P. agglomerans*) disappearing as they are diluted out of PCR contention by the more numerous LAB copy numbers. The disappearance of *P. agglomerans* bands in silage DGGE analysis has been previously reported by Wang and co-workers in as little as 2 d for alfalfa silage dominated by inoculated *L. plantarum* and after 20 d when colonised naturally by *L. sakei* and *L. kimchi* (Wang *et al.*, 2006). It is worth mentioning the bacteria not producing significant bands in DGGE but having a presence in enrichment dependant plating experiments. *Leu. mesenteroides* is a non-spore forming LAB that occurs epiphytically and initiates fermentation in a variety of foods and feeds including sauerkraut and silage (Thunell, 1995). *Leu. mesenteroides* is not acid tolerant and growth only occurs above pH 5.4, below which the silage is superseded by *Lactobacillus* sp. which are more effective at reducing proteolysis and spoilage of the feed (Adesogan *et al.*, 2003). *H. alvei* are gram negative, non-spore forming, facultative anaerobic, enterobacteria found to colonise

recently clamped grass silage (Heron *et al.*, 1993) where they outcompete existing populations of *P. agglomerans* (Östling and Lindgren, 1995).

Variations in the microbial community composition due to chemical and heat treatment or inoculation with *L. buchneri* were well highlighted compared with the control and *L. plantarum* fermentations, which were found to be similar to the fermentation without intervention. It is also apparent that the addition of LAB to unsterilised material prior to incubation does not yield a final population consisting of the isolate alone. Instead it is evident from the current results and in many other studies that final counts of both LAB and yeast may be more attributed to initial conditions in the silo such as temperature, oxygen levels, moisture content, availability of sugars, pH and presence of microorganisms and metabolites (McDonald, 1981, Jard *et al.*, 2011, Dunière *et al.*, 2013). Perhaps more importantly in terms of formation of stable silage is the succession of LAB from facultative homofermentative strains such as *L. plantarum* to obligate heterofermentors such as *L. buchneri*. *L. plantarum* swiftly converts water soluble sugars to lactic acid and thus avoiding the proliferation of unwanted organisms such as Clostridiaceae, Enterobacteriaceae and yeast by dropping the pH, which also prevents the loss of excessive dry matter and protein-lysis in the silage (Driehuis, 2013, Virtanen, 1945). *L. buchneri* is considered a late stage ensiling LAB as it converts lactic acid to acetic acid, a more potent biological preservative which increases the aerobic stability of the silage by inhibiting yeast and mould when the silo is opened (Holzer *et al.*, 2003). *L. buchneri* is often found to be the dominant LAB in mature silage due to its resistance to low pH. For these reasons *L. buchneri* is often inoculated with a homofermentative LAB such as *L. plantarum* or *L. pentosus* (Arriola *et al.*, Filya, 2003).

The role of *L. buchneri* illustrates how metabolic diversity is as relevant as species diversity when concerning the final metabolite make-up of the silage and the feed integrity as a whole. The metabolites produced by one pathway/species can sustain another, the kinetics of which being dependant on oxygen saturation, pH and temperature (Karlovsky, 2008). The same inter-organismal processes may be responsible for DON degradation within the silo; hence the importance of testing mixtures as well as single isolates as it is possible that one bacteria species performs one aspect of the degradation with another completing the task. Conversely, the metabolites produced by LAB may hinder the bacterial groups where DON interaction is a possibility. The current results suggest silage colonised by productive LAB or preserved with Crimpstore® do not exhibit a decrease in DON, whereas the heat treated samples which contained very different assemblages and metabolic diversities, reduced DON considerably. On one level this provides an opportunity for more selective screening of potential DON-degraders, but on another is gives an insight into possible the fate of this resilient molecule in the crop debris/soil environment and reveals one reason why it does not readily accumulate in the soil (Völkl *et al.*, 2004).

The advantage of using low volume silos is that multiple sampling to gain an idea of homogeneous species distribution is not required, as the entire mesocosm is extracted for DNA and any spatial differences in species richness within the silo make up the common pool of DNA. Larger scale silos require robust sampling techniques to monitor silage communities, as different regions of the silo with varying aerobic properties and crop quality may favour specific groups (Li and Nishino, 2011). One aspect the current study does not address is how the community changes over time, the pace of change and

which period yields the most significant reduction in DON. Temporal fluctuations in community succession and DON concentration could be recorded in future incubations by undertaking a time-course experiment with an extended incubation time, in line with other silage/DON studies such as Boudra *et al.* (2008) who monitored maize silage right up to 9 months. The CG bacterial community characterisation in the current investigation aids in the development of future DON-interacting inoculants. Firstly, it is apparent that silos with a reduced recovery of DON were colonised by a particular group of potentially DON-degrading taxa, some which will be isolated as live cultures using selective media and tested in the following chapter. Secondly, it is clear that should an isolate possess DON-degradation properties, before it can be used as a silage additive it would need to have a proven ability for survival, niche development and DON-degradation efficacy within the fiercely competitive silo environment, as illustrated with the current results.

5 Testing potential DON degrading isolates *in vitro* and in *F. culmorum* contaminated crimped barley and wheat fermented in mini silos

5.1 Aims and objectives

Develop an *in vitro* DON degradation assay to screen candidate isolates over short and intermediate exposure durations with actively dividing and static bacterial cells. The same strains will be inoculated into ensiled crimped barley and wheat previously infected with DON-producing *F. culmorum* as a more realistic remediation scenario.

5.2 Introduction

CG silage fermented with LAB forms a large proportion of European cattle total mixed ration (TMR) (Finch *et al.*, 2014) , although a good quality silage is often dependant on the quality of the starting material, *i.e.* moisture and carbohydrate content, MT contamination and inoculum presence. Whilst a lot of work has focussed on the development of CG silage LAB and yeast additives (Adesogan *et al.*, 2003, Olstorpe *et al.*, 2010b) for producing consistent fermentations, little attention has been shown to testing cereal epiphytic isolates for both the ability to degrade MTs as bio-control agents (BCAs) and partake in adequate CG fermentation. The use of BCAs is a rapidly expanding research area due to the need for effective fungal pathogen treatments which yield food and feed free from MT contaminants, antibiotics and fungicidal residues (Pal and Gardener, 2006). BCAs offer a self-perpetuating solution to selected agricultural issues, including the inhibition of MT-producing moulds in the field (Dalie *et al.*, 2010) and degrading MTs in stored produce (Hawar *et al.*, 2013).

Recent developments in terms of DON amelioration by beneficial bacteria/BCAs have been made by Ito and co-workers (2012, 2013) who applied a DON-metabolising bacteria

isolated from FHB-infected wheat heads to harvested contaminated material and recorded a 66% reduction in DON when incubated with the addition of the surfactant, Tween-80. The bacteria tested in this final chapter were also isolated from a matrix where DON could reside naturally, and where DON recovery was significantly reduced, and therefore could potentially be the biological agents responsible for this decrease. Whether DON could be subjected to interaction with any of these bacteria, either by binding or metabolism, is investigated in the current chapter, firstly *in vitro* then in a mini silo scenario. The bacteria should be able to survive and proliferate in such a matrix as they were isolated from a similar environment originally. This property could be valuable considering the application, whereas other bacteria with DON remediation ability would be physiologically unsuitable for fermented feed, *i.e.* aquatic sources (Völkl *et al.*, 2004).

The strains chosen for further *in vitro* and in silo testing include prominent bacterial groups determined in DON degrading assemblages deemed to be non-pathogenic, have an ability to culture readily under laboratory conditions and be relevant for the application of fermented grain feeds. *Pantoea agglomerans* (Enterobacteriaceae) is currently applied as a bio-control agent (BCA) in orchards and fruit packing houses (Nunes *et al.*, 2002), and whilst some strains are potentially opportunistic plant and human pathogens (Cruz *et al.*, 2007), the reports where *P. agglomerans* has made a large proportion of microbial mass in Scandinavian grain destined for ensiling (Olstorpe, 2008) and the BCA potential, makes it an interesting candidate bacteria to test.

Another bacteria present worth pursuing is *Weissella cibaria,* a heterofermentative LAB also found to be effective as a BCA in pomology, through the inhibition of *Penicillium expansum*, the mould responsible for patulin production (Trias *et al.*, 2010). In a multi-

farm screening of candidate ensiling bacteria, *W. cibaria* made up 34.4% of maize silage bacteria (average of 5 locations in Beijing) and the silage at 9 months was considered high quality (Pang *et al.*, 2011). *W. cibaria* is also present in Scandinavian CG silage, although in common with *Leuconostoc mesenteroides*, it is usually only abundant in the early stages of CG fermentation (Olstorpe *et al.*, 2010a). *Leu. mesenteroides* is a LAB with GRAS status used in the production of sauerkraut among other food-grade fermentations. *Leu. mesenteroides* also gave ensiling results comparable, but slightly inferior to *L. buchneri* in terms of DM losses when inoculated into crimped wheat (Adesogan *et al.*, 2003). *L. buchneri* is included in this study to act as a positive control for high quality fermentation, and a negative control for DON interaction. *Hafnia alvei* (Enterobacteriaceae) has previously been trialled as a BCA against *Fusarium* wilt following isolation from the rhizosphere of the affected plants (Sneh *et al.*, 1985), although the work focused on conidia germination rather than degradation of virulence compounds. When comparing between the other Enterobacteriaceae sp. found in grass silage during a typical fermentation, *H. alvei* was found to proliferate swiftly within the first 4 d and outcompete the initially dominant *P. agglomerans*, although once the LAB fermentation is initiated all gram negatives decline rapidly (Östling and Lindgren, 1995).

The current chapter involves firstly characterising these isolates in terms of growth and *in vitro* DON-metabolism. This is designed to screen for and define potential detoxification mechanisms. Secondly, a more robust proof of concept experiment is carried out by testing isolates in a more realistic CG scenario in mini-silos as used previously. The grain used for ensiling will be from in house grown wheat and barley infected with a toxigenic F.

culmorum strain under greenhouse conditions to produce a naturally DON-contaminated matrix for detoxification.

5.3 Materials and methods

5.3.1 Verification of DON conjugation in grain used for crimping and generation of contaminated grain for degradation studies

Barley (*cv.* Westminster, spring) was planted in sterilised loam in 1 L pots under unheated greenhouse conditions at 200 plants/m^2. At anthesis the barley heads were inoculated with *F. culmorum* (see below) for 21 d with moisture levels kept high using polythene bags (200 µl of 10^4 macro-conidia / ml / barley head). The *F. culmorum* (309344) strain was obtained from CABI (UK) and genotyped for the trichothecenes and DON producing genes using the Tox5-1/Tox5-2 (Niessen and Vogel, 1998) and Tri13F/Tri13DONR (Chandler *et al.*, 2003) primer pairs for identifying trichothecene and DON producers respectively using the PCR program of Kammoun *et al.* (2010) and visualisation using a 45 min electrophoresis of product in 1% agarose stained with SYBR safe[(R)]. *F. culmorum* was maintained on SNA-FP (Spezieller Nährstoffarmer Agar-filter paper) or on autoclaved barley for spore production in 500 ml Erienmeyer flasks (50 g of 12% moisture barley autoclaved twice at 110 °C / 25 min, adjusted to 40% w/w moisture with sterile H$_2$O, inoculated with 10^4 conidia / ml 7% NaCl / 0.05% Tween-80 harvested from agar and incubated for 7 d at 25 °C). Five days before harvest a group of 20 non-inoculated barley plants were spiked with purified DON (50 µl of 50 µg/ml per barley head) and grown in a 20 °C incubator (16 h light). At 21 d, the grain was harvested and the grains processed by hand. The barley was at the "soft cheese stage" and the wheat was "hard cheese stage", slightly more mature. IAC-HPLC analysis was undertaken as stated previously to confirm

the presence of DON and D3G. Wheat (*cv.* Claire, winter) was also inoculated and treated in the same way as a comparison (in 30 cm x 30 cm x 25 cm pots) and to generate contaminated material for future ensiling experiments.

Table 5. 1. Primers employed for determining the presence of *Fusarium* sp. virulence genes.

Primer specificity	Forward	Reverse
Tox5-1/Tox5-2 for determination of trichothecene producing ability	Tox5-1 (5'-GCTGCTCATCACTTTGCTCAG-3')	Tox5-2 (5'- CTGATCTGGTCACGCTCATC-3')
*Tri13*F/*Tri13*DONR for determination of DON production ability	Tri13F (5'- CATCATGAGACTTGTKCRAGTTTGGG-3') (K: A or G, R: G or T)	Tri13DONR (5'- GCTAGATCGATTGTTGCATTGAG-3')

5.3.2 Growth curves of each strain with different inoculation amounts

Strains identified as potential DON-degraders in the last Chapter for further screening were inoculated from frozen stocks (TSB, 10% DMSO at -80 °C) into 50 ml TSB (tryptone soya broth) in pre-weighed 50 ml centrifuge tubes and incubated at 30 °C in 2.5 L anaerobic jars (Oxoid, UK) with 1 Anaerogen™ sachet for 24 h. Cultures were centrifuged (10000g, 10 min) and washed with PBS and the cell pellet and tube weighed in order to calculate the number of bacteria present (determined with pellet weight/plating out calibration curve experiments where serial dilution of confluent broth were centrifuged and weighed, then resuspended in 1 ml PBS and enumerated on TSA) which was then diluted to 10^6 CFU/ml by addition of minimal salts medium (MSM). Six candidate strains were inoculated at different cell densities into 500 µl TSB as 10 µl volumes in a 48-well plate (Nunc, UK), flushed with nitrogen and sealed with parafilm™. The plate was incubated at 25 °C in an Infinity-200 plate reader (Tecan, Austria) for 144 h with readings every 30 min.

5.3.3 Preliminary *In vitro* DON degradation experiment

A 24 h culture was obtained by sub-culturing each strain into 50 ml of TSB broth (100 µl of confluent inoculum) and incubating anaerobically at 25 °C in a pre-weighed 50 ml centrifuge tube. The tube was centrifuged (10000g/10 min) to yield a pellet that could be weighed and the cell count determined indirectly. The pellet was re suspended in MSM to 10^{10} cells/ml and divided into 500 µl microcentrifuge tubes (with holes in the lid to permit anaerobic condition to occur, made with 21G hypodermic needle) which were centrifuged (10000g/5 min) and the supernatant removed and replaced with either MSM or TSB containing 50 µg/ml DON. The tubes were then incubated at 25 °C under anaerobic conditions for 24 h and 2 wk without shaking. Tubes were then centrifuged (10000g, 10 min) and the supernatant filter sterilised (0.22 µm) into a micro HPLC vial (250 µl, Fisher) and capped. The *P. agglomerans* treatment was not easily filtered, so required the additional use of a 30,000 MW molecular sieve (Sigma) centrifuged at 3000g for 20 min. 50 µl of sample was injected into the HPLC with the DON gradient program as used previously. The DON integrated peak area was compared between the bacterial incubations and the sterile control treatments. Any metabolites were compared to injected standards that included; 3AcDON, 15AcDON, DOM-1.

5.3.4 *In vitro* DON degradation time course experiment

Strains exhibiting reduction in DON recovery in the 24 h and 2 wk exposures tested again in a more comprehensive time course experiment. To prevent the loss of anaerobic conditions during sampling all experiments were undertaken in 5 ml syringes (Gillette, UK) with a sheathed 21 gauge hypodermic needle (blunt) to allow sterile sampling by expelling contents into microcentrifuge tubes for processing as stated above. A latex gas balloon filled with nitrogen attached to a piece of silicone tubing and blocked one end

served as a way of adding 0.5 ml of nitrogen gas to enable mixing of syringe contents prior to sampling. A syringe needle pushed diagonally into the tube and withdrawn would allow the tubing to reseal. Bacteria were suspended in MSM with 50 µg/ml DON at a density of 10^{10} cells/ml. All syringes were placed in anaerobic jars between samplings and inverted twice daily (Figure 5.1). Five hundred µl of each sample expelled was serially diluted in DPBS and 20 µl of each dilution from 10^{-3} to 10^{-10} was dropped onto quartered TSB plates and incubated anaerobically at 25 °C before enumeration.
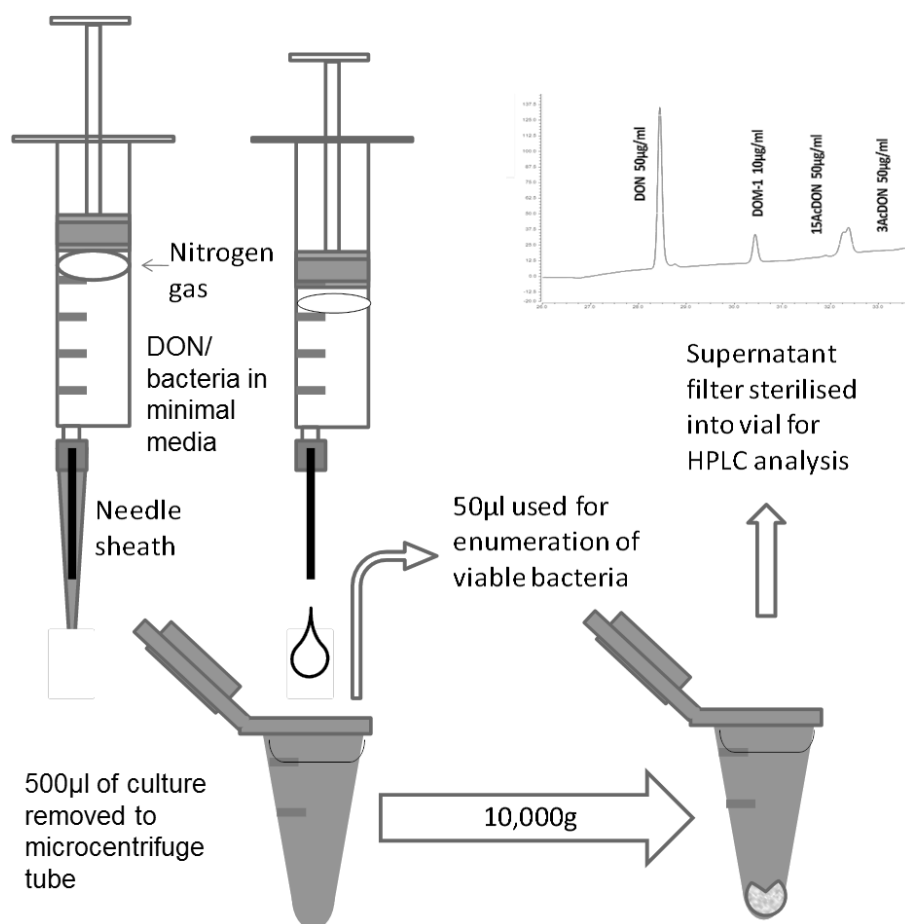
Figure 5. 1. Diagram of sampling processes for anaerobic time-course experiment. The provision of nitrogen gas in the syringe allowed mixing of contents prior to sampling without compromising anaerobic conditions. All syringes were placed in an anaerobic jar between sampling and inverted daily.

5.3.5 Testing of candidate bacteria for DON reduction in *Fusarium* enriched crimped wheat and barley grain.

In order to verify the DON-reducing capacity of *Weissella cibaria*, *Hafnia alvei* and *Pantoea agglomerans* a final ensiling experiment was undertaken as in chapter 3 with some important modifications. The grain employed was more realistically contaminated with DON (Figure 5.2) rather than spiked with a standard (the Barley was diluted with uncontaminated grain at ~40% moisture to provide enough material for the experiment, which was additionally spiked with DON (to make ~4 µg/g moist grain). The grain was then gamma irradiated (Co^{60} 25 Gy, Becton-Dickinson, Plymouth) in individually weighed aliquots in polythene bags (~50 g Barley, ~35 g Wheat) and then frozen until ensiling (-20 °C). The grain was thawed, "gently crimped" with a sterile mortar and pestle in a laminar flow hood (Bassaire, UK) and compressed into either 50 ml polystyrene pots (Barley) or within the sealed bags (Wheat).

Figure 5. 2. Culture, inoculation and crimping of wheat and barley for determination of DON conjugation capacity and to generate naturally contaminated material for future degradation experiments. A; Spores harvested from *F. culmorum* (309344) cultured on sterile barley, B; glass house grown wheat inoculated with *F. culmorum* spores with polythene bags increasing humidity therefore aiding colonisation, C; crimped barley prior to ensiling.

The cultures were added at 10^5 CFU/g moist CG as before with the grain 35-40% moisture. In addition, a mixed treatment consisting of *W. cibaria*, *H. alvei*, *P. agglomeran*s and *L. buchneri,* each inoculated at 2.5 x 10^4 CFU/g CG. This essentially served as a check to see if DON levels are more easily reduced in a more realistic assemblage/metabolic diversity scenario. *L. buchneri* was also tested in isolation as a positive control for silage integrity purposes. Once sealed, the triplicate mini-silos were contained in sealed plastic boxes and incubated at 25 °C for either 2 or 4 weeks before extraction in DPBS as described previously, enumeration on TSA plates (anaerobic, 25 °C, 48 h), lactic/acetic acid analysis and IAC-HPLC for determination of DON and D3G recovery differences from the time-zero control.

5.4 Results

5.4.1 Evaluation of *F. culmorum* 309344 virulence using genotyping

Following PCR of extracted *F. culmorum* DNA it was apparent that although both 309344 and the non-toxigenic strain were positive for trichothecene synthesis pathway genes (650bp), only 309344 was positive for the gene responsible for DON production (282bp). Genotyping can only indicate the potential for these strains to produce these MTs, and Reverse transcriptase-PCR / actual enzyme activity / measured MT production / effect of plant growth would be required for confirmation of virulence (Figure 5.3).
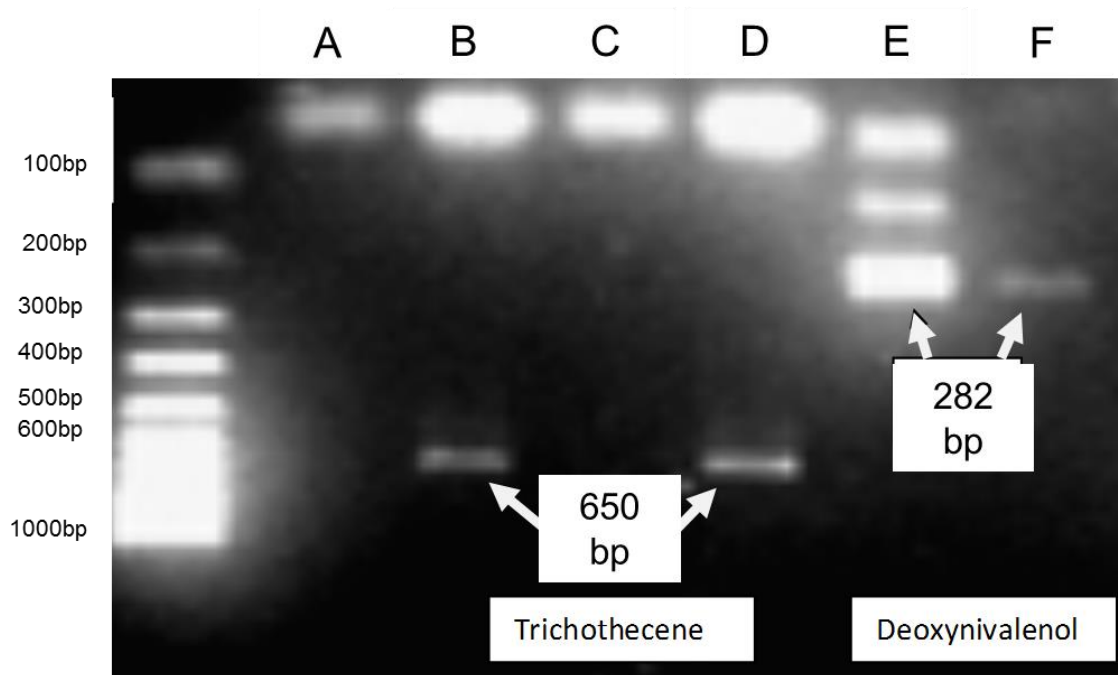
Figure 5. 3. Confirmation of *F. culmorum* toxin producing ability using PCR of key trichothecene (Tox5, 650bp) and DON (Tri13, 282bp) genes. Lane A; Tox5 toxigenic strain 309344, B; Tox5 309344 repeat pcr, C; Tox5 Non-toxigenic strain, D; Tox5 Non-toxigenic strain repeat PCR, E; Tri13 309344, F; Tri13 Non-toxigenic strain.

5.4.2 Verification of DON and D3G production in barley used for crimping

The ability of the barley cultivar (Westminster) used in the current study to conjugate DON to D3G was illustrated following exposure of growing immature barley kernels to the purified DON (21.9% was D3G form), yet the *F. culmorum* 309344 infected grain yielded extremely low levels of D3G (<0.1% D3G) even though levels of DON were similar to that spiked with the purified toxin (Table 5.2, Figure 5.4). Since the exposure periods differ, it is difficult to state whether the DON found with *F. culmorum* was because of accumulation over the 21 d or over a much shorter period. It is possible that a mechanism exists where the mould is able to de-conjugate D3G as an additional virulence pathway whilst colonising the grain. Further work is required to assess this possibility. It is equally possible that a weak plant is unable to yield sufficient conjugation products to be measured and a healthy plant exposed over a much shorter time has a greater ability for detoxification of DON. Since the *F. culmorum* infected grain was used for the final

experiment, it is important to note that no D3G was detected for either commodity before or after ensiling. This suggests the levels of D3G were not sufficient to contribute to the overall level of DON.
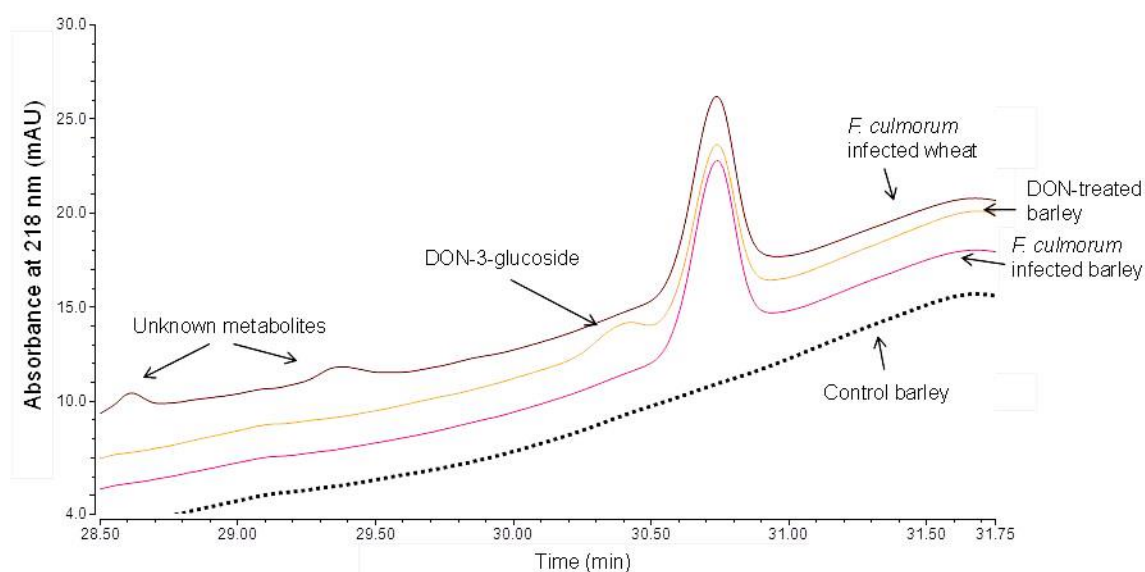
Figure 5. 4. Comparison of DON and D3G levels in barley and wheat infected with *F. culmorum* (anthesis-14 d) or DON-treated barley heads (anthesis- 7 d)

Table 5. 2. Summary of DON and D3G yields *in planta* following two methods of contamination. Results from pooled samples.

Barley treatment	Incubation time	Concentration of DON	Concentration of D3G (% of total yield)
Inoculated with toxigenic *F. culmorum*	21 d	637.1 µg/kg	0.5 µg/kg (<0.1%)
Spiked with DON standard	7 d	533.4 µg/kg	149.5 µg/kg (21.9%)

5.4.3 Growth curves of each strain in broth with different inoculation rates
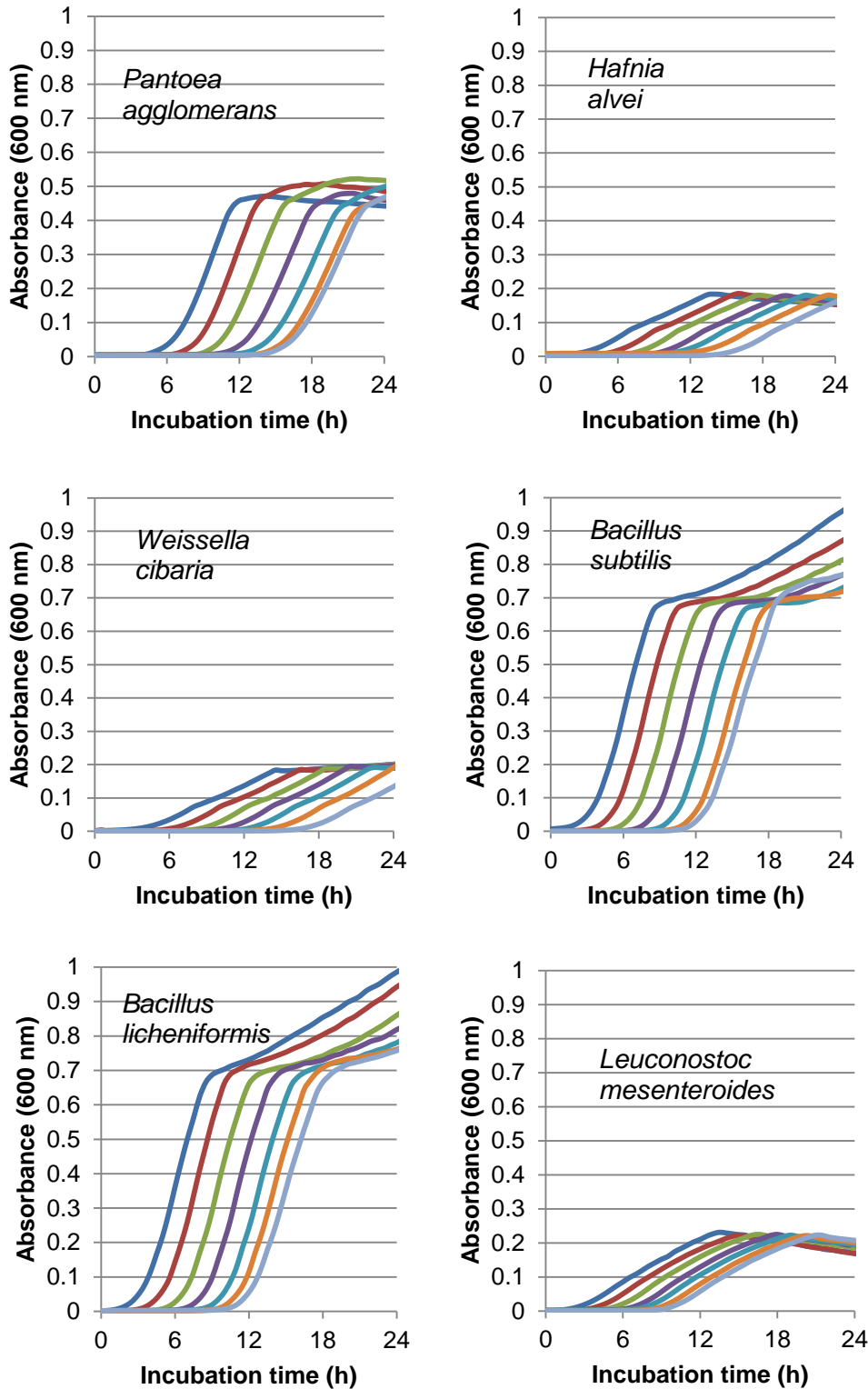
Figure 5. 5. Comparison of representative growth curves and inoculation concentration of *Pantoea agglomerans*, *Hafnia alvei*, *Weissella cibaria*, *Bacillus subtilis*, *Bacillus licheniformis* and *Leuconostoc mesenteroides* (10µl inoculant as $\log_{10}$ dilution series from 2.0 x 10^8 to 2.0 x 10^2 CFU / ml) incubated in tryptone soya broth for 6 d at 25 °C and the absorbance read every 30 min at 600 nm.

In general the growth curves aid in confirming the relatedness of the six strains in terms of proliferation characteristics (figures 5.5). It is evident that the *Bacillus* sp. have homologous curves and similar maximum rates of absorbance change, suggesting that growth characteristics between similar species are not sensitive enough for identification purposes alone. *W. cibaria, Leu. mesenteroides* and *H. alvei* were found to not differ significantly in regards to the maximum growth rate and formed the same cluster, yet *P. agglomerans* was significantly different to these and the *Bacillus* sp. (ANOVA, F = 794.2, <0.001, n = 42). Logarithmic growth generally reaches a plateau phase within 24 h, therefore sub-culturing for experiments was undertaken every 24 h. Whilst there was a lag phase linked to the inoculant density, it did not affect the maximum population growth rate.

5.4.4 *In vitro* degradation experiments

Spike and recovery experiments in DPBS, MSM and TSB were undertaken to verify that TSB could be used for a degradation experiment providing a sufficiently high DON concentration was used (50 µg/ml, visual evaluation of chromatogram), although DPBS/MSM gave less chromatogram interference (Figure 5.6). The HPLC method employed for determination of DON in media was very effective for separation of possible DON-metabolites such as deepoxyDON (DOM-1), 3-AcetylDON, 15-AcetylDON and potential homologous structures (Figure 5.8).
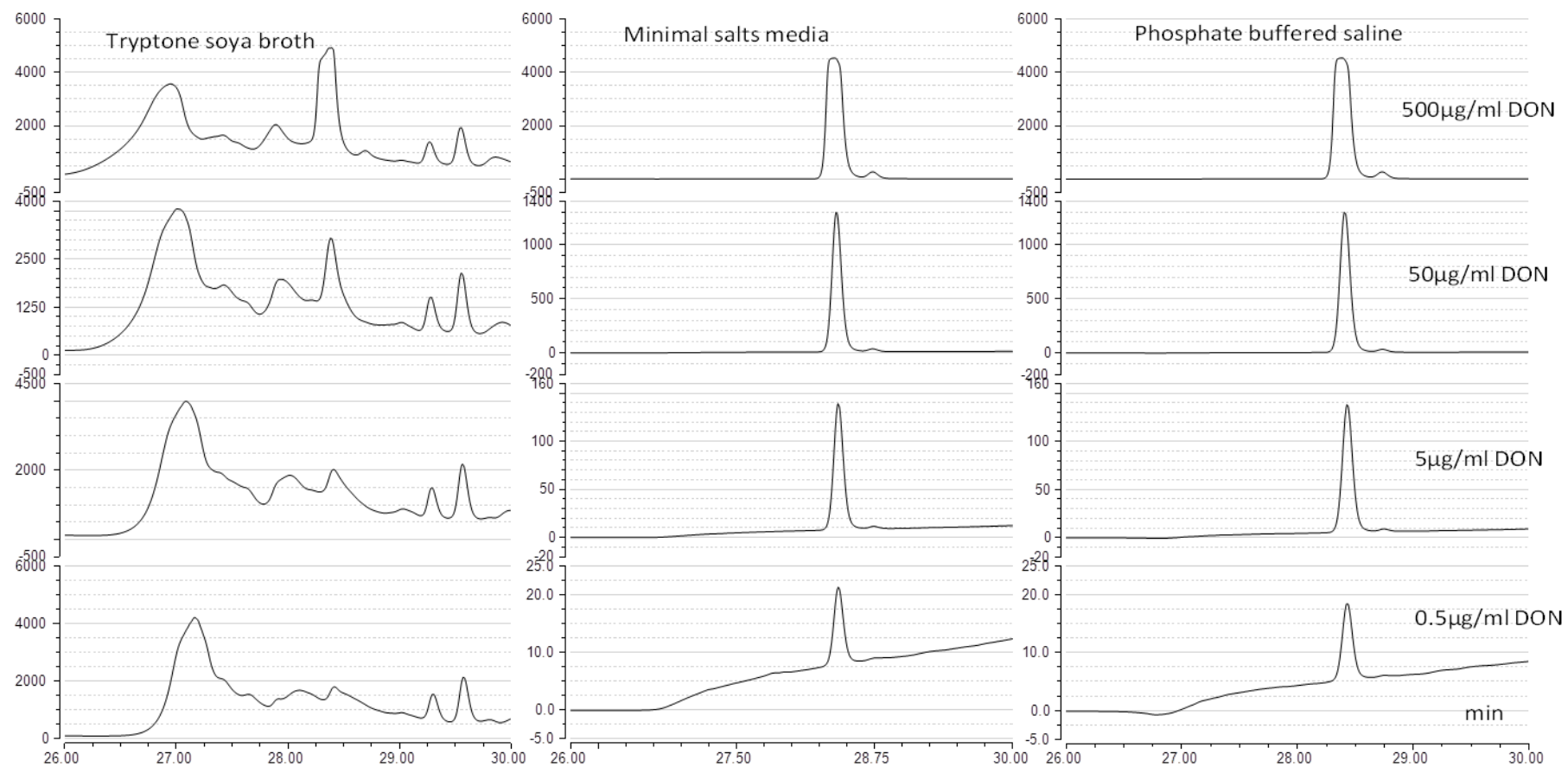
Figure 5. 6. The identification and measurement of different concentrations of DON in tryptone soya broth, minimal salts medium and phosphate buffered saline (Dulbecco's A).
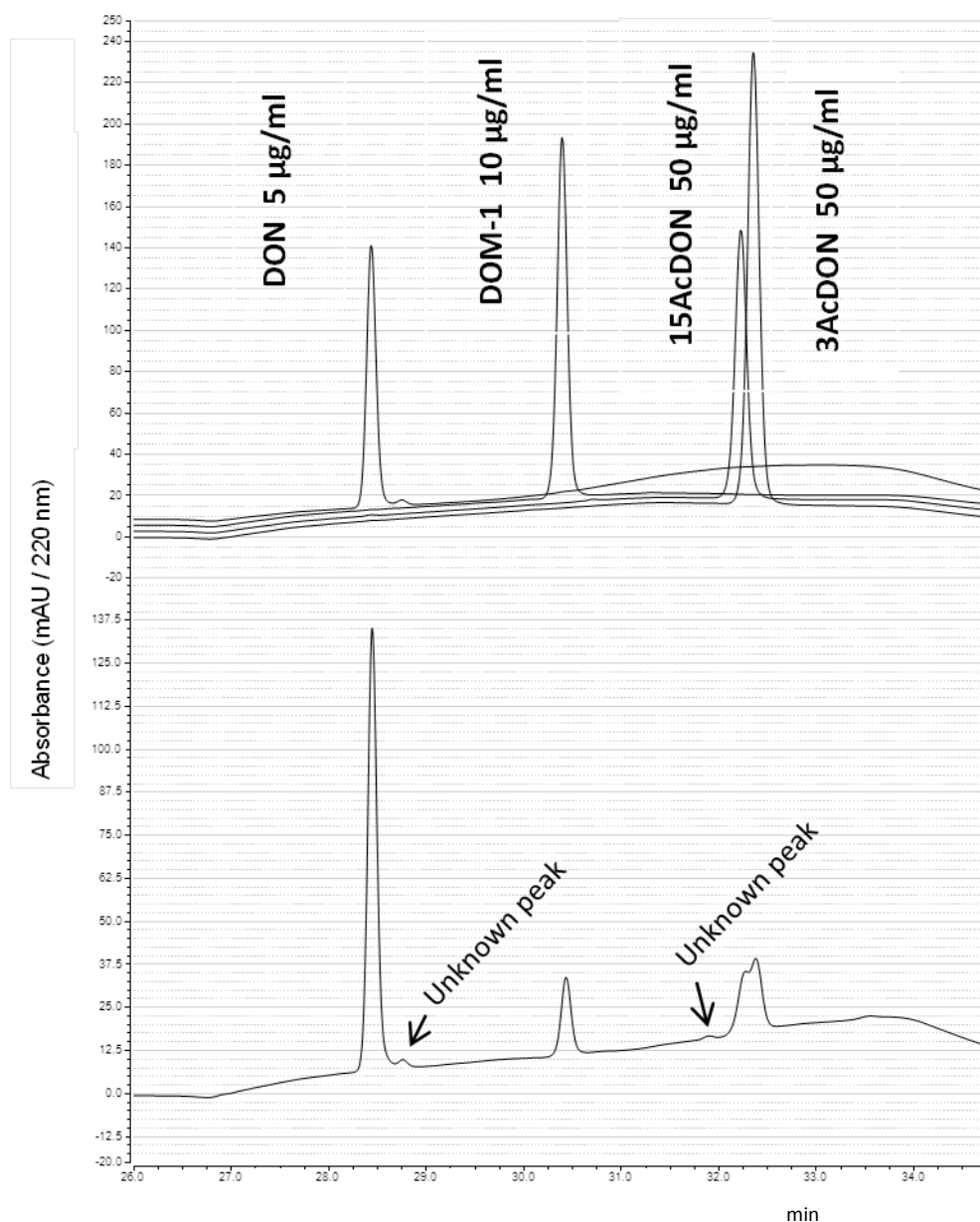
Figure 5. 7. HPLC analysis of a range of DON metabolites that include DON, DOM-1, 3AcDON, and 15AcDON showing adequate separation required for degradation studies. Some unknown peaks were also present, perhaps degradation products of the standards.

The percentage recovery of DON following static 24 h incubation of 10^{10} bacteria indicated that all treatments showed a reduction in recovery over the control, although after 2 wk; *P. agglomerans, B. licheniformis* and *Leu. mesenteroides* treatments returned to control DON recovery levels (Figure 5.8, 5.9). *B. subtilis* produced a variable response with *H. alvei* and *W. cibaria* reducing DON recovery significantly (11.3 and 6.2% recovery, $p < 0.05$, ANOVA). The same incubation in TSB with presumably proliferating bacteria

yielded chromatograms with too many additional peaks to decipher DON content and was abandoned.
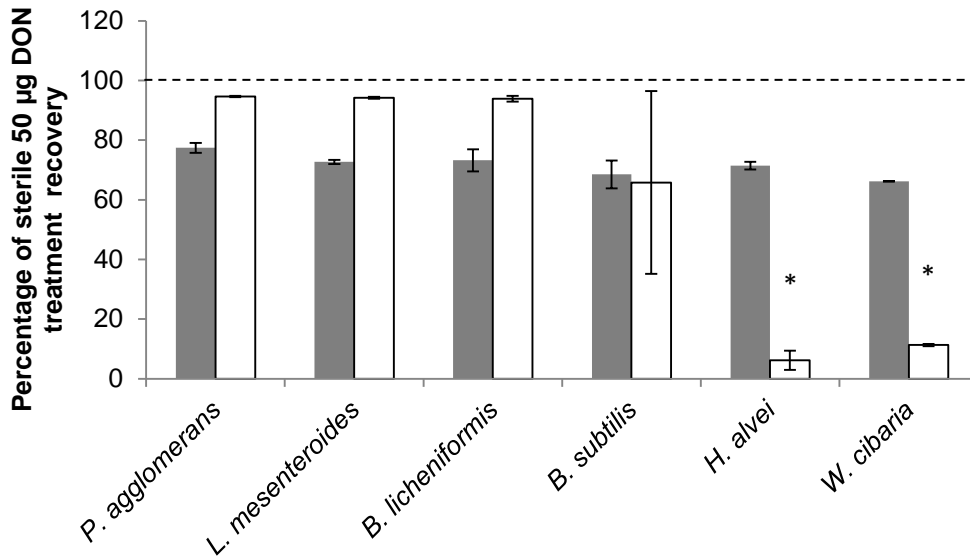
Figure 5. 8. Percentage recovery following a 24 h (grey) and 2 week (white) incubation at 25 °C of 6 bacterial species at a density of 10^{10} cells/ml to 50 µg/ml DON in MSM (triplicate tubes).
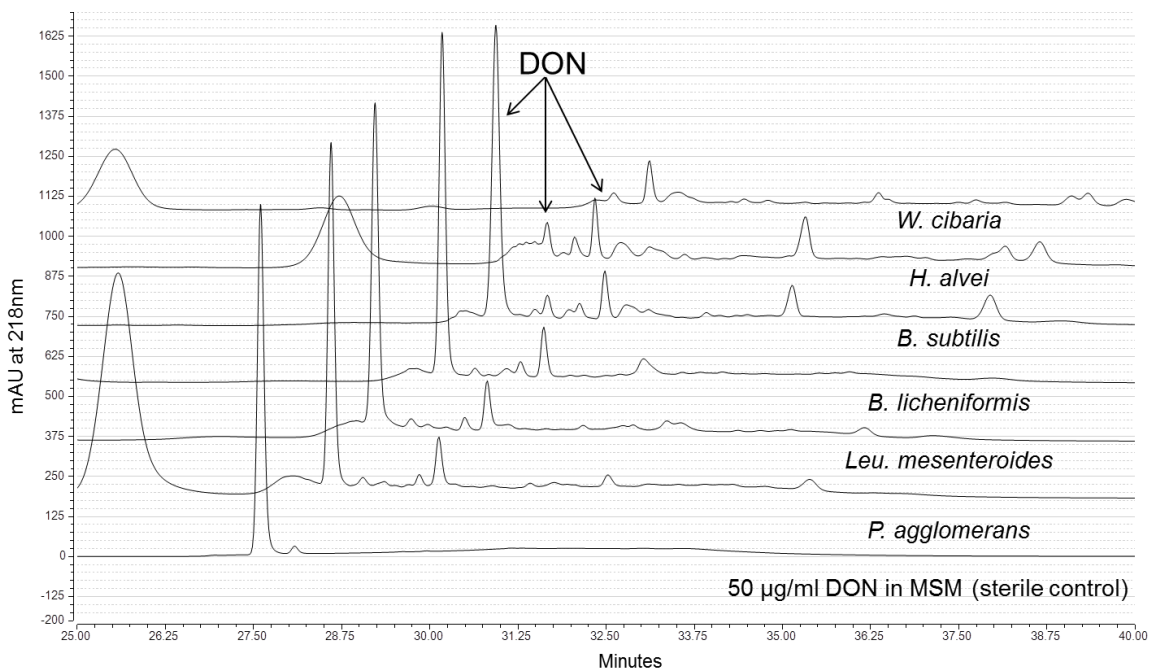
Figure 5.9. Stacked and time-offset chromatograms of 50 µg/ml DON in MSM incubated for 2 wk different bacterial inoculations or left sterile. Each chromatogram is representative of triplicate incubations.
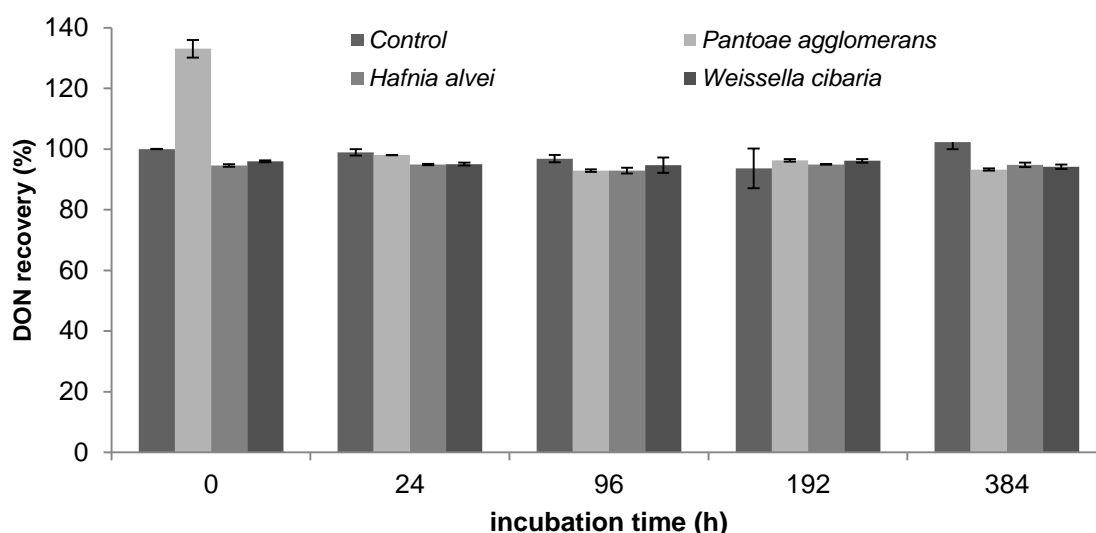
Figure 5.10. Time course DON degradation experiment in MSM. Bacteria were suspended in minimal medium containing 50 µg/ml DON at a density of 10^{10} cells/ml and incubated for up to 384 h at 25 °C under anaerobic conditions in 5 ml syringes. The mean of triplicate syringes is shown with the standard error.

A more comprehensive time-course degradation experiment was devised which would enable sampling to take place without compromising anaerobic conditions. Even though cell counts were extremely high initially there was no reduction in DON recovery over the entire incubation (Figure 5.10). *P. agglomerans* produced the only deviation from the control with a 30% increase in DON concentration at time-zero following re-suspension of the cell pellet in the MSM. This was the same sample group which required alternative centrifugal molecular sieve filtration methods in preparation for HPLC due to the gelatinous hydrophobic extracellular polysaccharide exudate produced by *P. agglomerans* for the purpose of early colonisation of surfaces prior to biofilm synthesis (Sutherland, 2001, Amellal *et al.*, 1999). This prevented passage through a 0.22 µm syringe filter, even with extensive pre-centrifuging of supernatant.
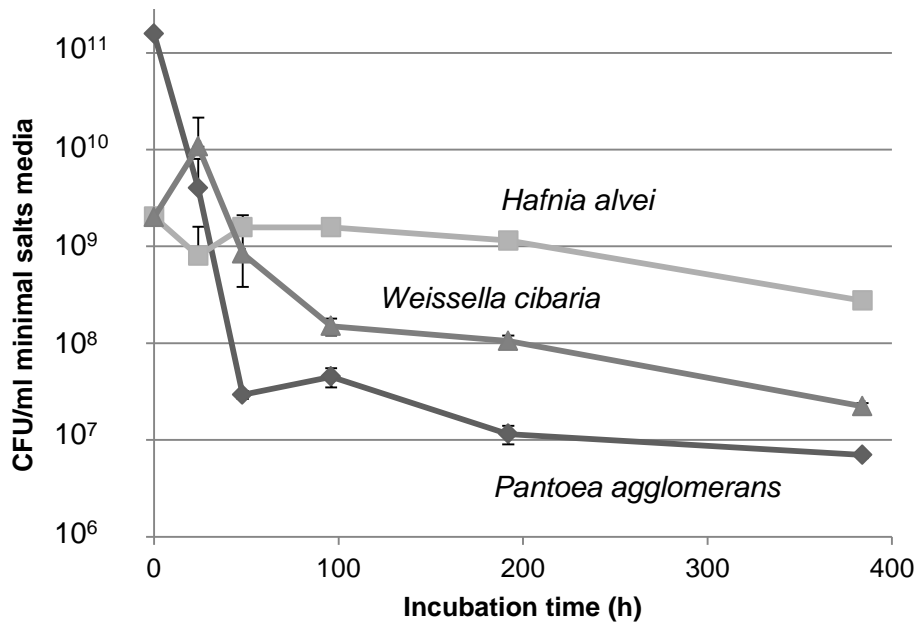
Figure 5.11. Enumeration of bacteria exposed to DON in MSM over time. Initial densities of ~10^{10} cells/ml were achieved by weighing a confluent pellet of cells and using a calibration curve. Mean of duplicate counts is shown with the standard error.

The viable counts for *H. alvei* and *W. cibaria* correspond well to the 10^{10} viable cells required initially (Figure 5.11). The *P. agglomerans* count was 100 fold more concentrated at time zero, but declined rapidly over 48 h followed by modest reduction in viable counts. At 384 h cellular viability for all species was still considerable at between 1.9×10^7 and 1.5×10^9 CFU/ml with *H. alvei* CFU counts reducing only slightly throughout the experiment. This result does suggest binding or biodegradation of DON does not occur within this timeframe without the substrates available for growth. This conflicts with the earlier experiment where DON removal was evident, suggesting a potential physiological difference between the bacteria in the two *in vitro* experiments due to biological changes or subtle differences in growth conditions.

5.4.5 Crimped grain DON-recovery experiments

The naturally DON contaminated crimped wheat and barley experiments were designed to determine whether DON degrading bacteria could be used to degrade DON in a semi-

realistic mini-silo scenario. The CFU counts at 2 and 4 wk were similar in barley with pH

dropping for LAB treatments but less so for the Enterobacteriaceae which corresponded

to the acid content measured by HPLC (Figure 5.12). The difference in counts between 2-

4 wk showed a trend of constant counts for the LAB and a 10-fold drop in

Enterobacteriaceae counts.   The wheat showed a more significant drop in counts

between 2 and 4 wk although the pH and acid content were largely unchanged.

The controls for the wheat and some of the barley silos at 4 wk, which were supposedly

sterile, did happen to have a background count of bacteria or yeast, although from the

counts, pH and acids content they do not appear to be LAB. The frozen sample had low

levels of lactic and acetic acid implying a degree of fermentation had occurred during the

defrosting of the aliquoted grain during the gamma irradiation process.

Following verification of sample dilution requirements by observing when the peak area

changed in proportion to the dilution amount, it was decided that a 5-fold dilution in

addition to the 3 fold dilution in PBS during extraction (15x dilution in total) was

necessary for wheat samples to prevent quenching of the column.   A 3 fold (9x dilution in

total) dilution was necessary for barley. This would allow any deviation from the initial

DON level to be measured. The barley was actually within the capacity of the column,

although close to the limit (Figure 5.13). The effect of the irradiation process on the

depolymerisation of cellulose and the breaking of the D3G-glucosidic bond in the grain

was not evaluated, but may explain why D3G was not found in any of the current

experiments. Also, the biocidal effects irradiation was not 100% effective with some of

the   control   silos   yielding   colonies,   although   LA/AA   levels   were   low.
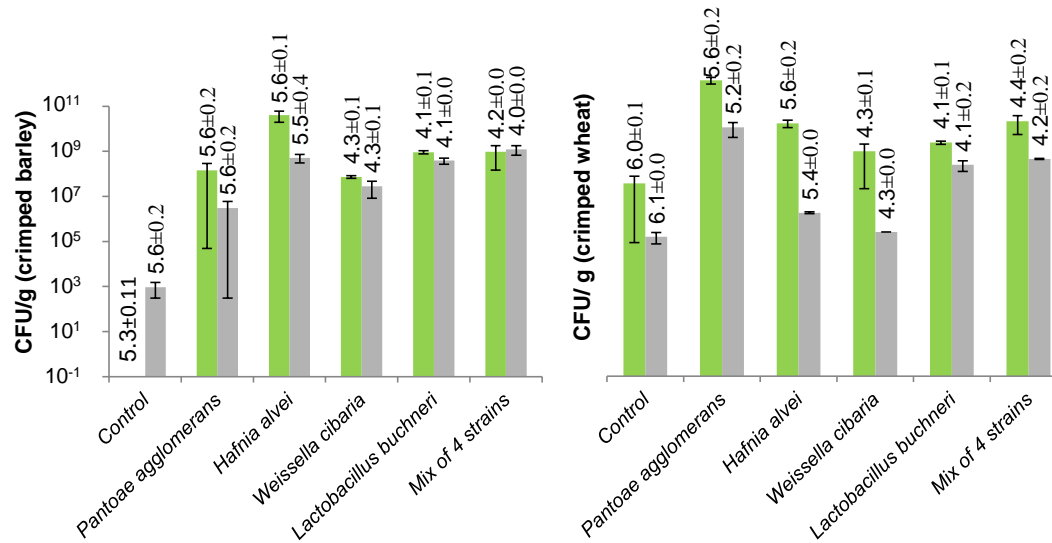
Figure 5.1 2 Crimped grain fermentation parameters measured in bacteria inoculated minisilos. Top: Viable counts on TSA (CFU/g) for barley (L) and wheat (R) over 14 d (green) and 28 d (grey) shown with the pH above.

Below: Lactic and acetic acid measurements for each treatment inoculant (mg/g moist grain) over 14/28 d with barley on L and wheat on R. All with SEM of triplicate minisilos.
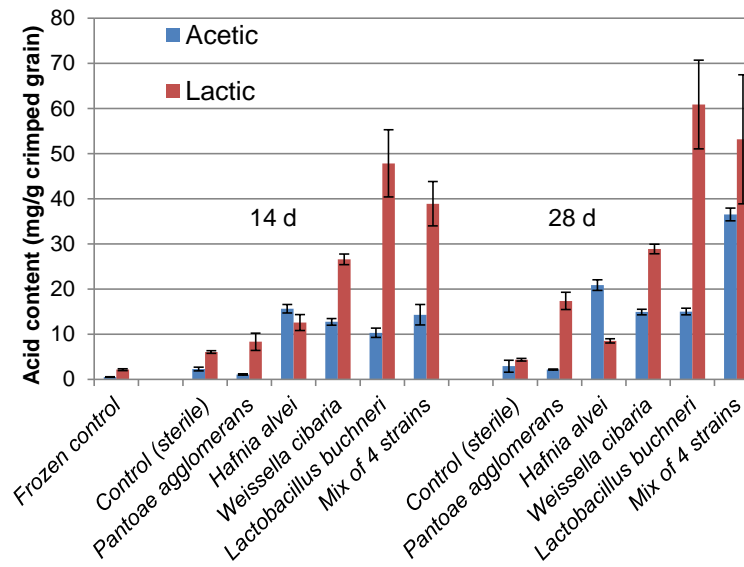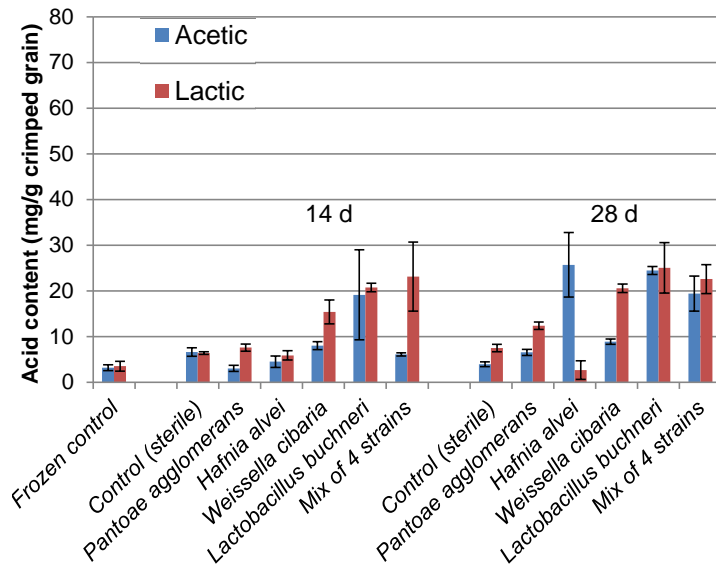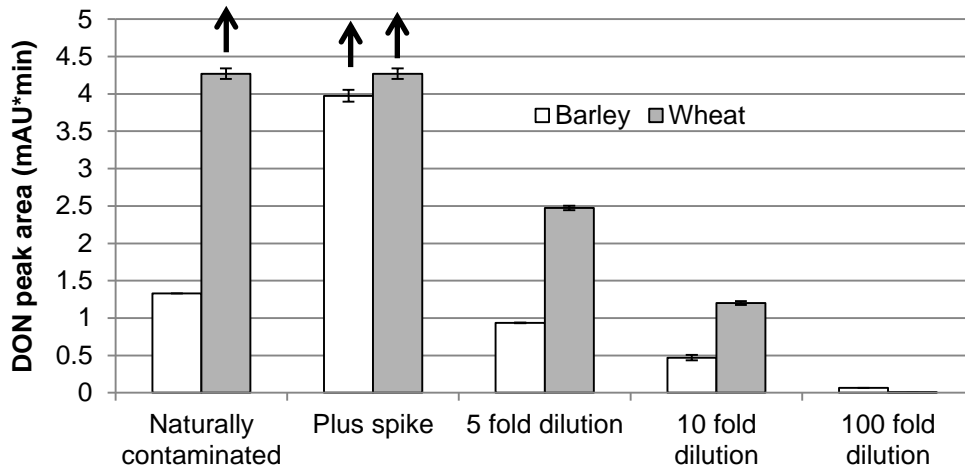
Figure 5. 13 Determination of sample dilution requirements before IAC purification. The mean peak area (mAU) of duplicate frozen wheat/barley samples is shown with the standard error. The arrows highlight where the recovery of DON was limited by the capacity of the column and is therefore an under-representation.
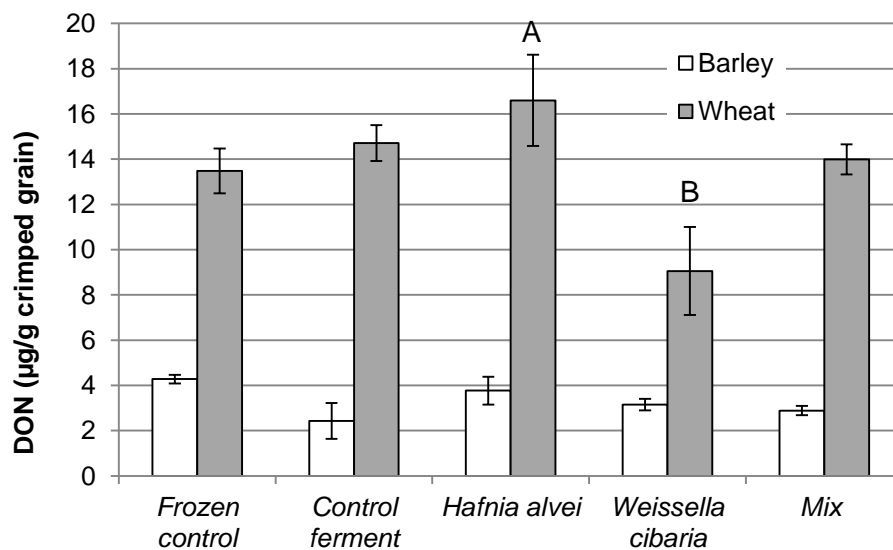
Figure 5. 14. Recovery of DON in naturally contaminated wheat and partially naturally contaminated barley following a 4 wk incubation with different inoculated silage bacteria under anaerobic conditions at 25 °C. The mean of triplicate silos is shown with the standard error. 'A' denotes a significant ($P<0.05$) increase in DON recovery over the other treatments whilst 'B' indicates a significant reduction in recovery.

Of the silos chosen for DON determination at 4 weeks, only the *W. cibaria* treatment in wheat showing a significant reduction in DON recovery compared with the other treatments including the frozen time-zero control (ANOVA, F = 3.92, *p* =0.036, Anderson-Darling statistic= 0.63, *P* =0.081, n = 15) with a mean reduction in DON recovery of

32.8±14.4% averaged from 7.0%, 34.7% and 56.7% for each mini-silo (Figure 5.14). This highlights the variability found with this complex matrix when dealing with inoculation of bacteria. *H. alvei* actually increased DON recovery over the frozen control which could be explained by this strain having the enzymatic capability to release locked up DON within the kernel without DON degradation. The barley data was suggestive of DON reductions in the *W. cibaria*, control fermentation and mixed treatments but not to a significant level. Furthermore, the barley did not produce the level of homo- and heterolactic metabolism seen with wheat even though the counts were comparable at 2 wk and with less of a decline at 4 wk.

5.5 **Discussion**

This aim of this chapter and the thesis as a whole was to isolate a biological agent able to degrade DON in a fermented feed scenario. Whilst more work is needed to characterise the nature of the interaction between *W. cibaria* and DON, the promising results measured *in vitro* and in the more realistic crimped wheat, reinforce the notion that a biological agent isolated from the agronomic environment and able to thrive in a fermentative context would be the most ideal solution to post-harvest DON-contaminated grain, or even as an epiphytic preventative pre-harvest treatment. Such an approach has already been successfully utilised by Ito *et al.* (2012) with a novel DON degrading Actinomycete isolated from wheat heads.

In the preliminary *in vitro* study, unmixed *W. cibaria* and *H. alvei* were shown to reduce DON, whereas in the time course sampling experiment the cells produced no such response, suggesting the interaction may not be as simple as metabolism of DON as a carbon source for survival. It is possible that co-metabolism, membrane binding or

internal compartmentalisation may be responsible for the decrease. Further experiments would optimise conditions for maximal effect whilst determining the exact nature of the interaction in broth and then within a contaminated grain matrix. It has been found by some workers that DON degradation can be transitory with the bacteria losing the ability under laboratory subculture (Shima *et al.*, 1997). Furthermore, some bacteria need to be primed before they perform certain metabolic tasks, a phenomenon discussed by Guan and co-workers (2008) who used a period of low nutrient culture with coumarin as a sole carbon source to induce AFB_1 degradation pathways.   Indeed, screening for DON degrading bacteria usually employs a sole carbon source approach (Völkl *et al.*, 2004), (although for sufficient growth a large amount of DON needs to be purified from contaminated media, which does have serious safety implications (Ito *et al.*, 2012)).

The DON co-metabolism theory may be supported by gene cloning work undertaken by Ito and co-workers (2013), who found DON to be effectively biotransformed to the lesser toxic 16-hydroxyDON by a redox pathway suite of enzymes and cofactors that include P450, ferredoxin reductase, ferredoxin and NADH. Unfortunately, in the current *in vitro* experiment there was little indication of the DON-biotransformation product DOM-1 or homologous structures which would give definitive evidence of interaction as was the case with patulin (Hawar *et al.*, 2013).

The possibility that binding is responsible for the decrease would require the pellet to be lysed and extracted as was achieved by El-Nezami and co-authors (El-Nezami *et al.*, 2002, El-Nezami *et al.*, 2004) with DON and ZEA using LAB. It must be questioned however as to why the second *in vitro* experiment did not yield any indication of binding whatsoever, even though the cell count was the same initially and the only differences being the

overall volume, the syringe delivery of sample and the daily mixing. It is possible that this mixing element be crucial to the reduction in DON-recovery in the supernatant. When the bacteria are allowed to settle for an extended period of time they can form a biofilm with polysaccharide and protein which either encapsulates DON as a form of binding, or provides a biological interface where DON can accumulate for enzymatic catabolism. The mixed time series samples did not have this stable period of bio-concentration and more work is required to characterise the exact mechanism, especially if binding is involved since DON will likely be re-released *in vivo* during digestion. Whether a binding mechanism is responsible for the reduction in DON recovery in the first experiment (chapter 3), or the reduction in the current wheat mini-silos requires further optimisation of the extraction process.

The difficulties faced with obtaining sufficiently DON-contaminated grain at the correct stage for crimping that could be used for larger scale experiments is logistically complex (hence the requirement for in-house grown and FHB-infected grain in the current study). Testing for DON contamination is usually carried out post-harvest on dried grains channelled into the food and feed industries. By this point the grain is beyond the stage where crimping can occur (although LF formulations can be employed for some production animals such as swine (Brooks, 2008)). In addition, the grain destined for use as home grown animal feed for cattle and sheep and harvested at 35-45% moisture is unlikely to be tested MTs, although a visual check and vigilant use of the HGCA risk assessment may indicate *Fusarium* MTs are present (HGCA, 2014). Furthermore, the ensiled CG is not likely to be tested prior to feeding, yet anecdotal and measured

evidence of feed refusal, reduction in animal productivity and behavioural changes may be cause for concern and worthy of further investigation.

Grain found to be DON-contaminated in the field would be an opportune subject for biotransformation experiments in a CG ensiling context where *W. cibaria* could be trialled under realistic conditions and with genuinely contaminated material. Whilst the focus would remain on DON, other co-occurring *Fusarium* MTs including D3G, 3AcDON, 15AcDON, Diacetylscirpenol, Nivalenol, T2/HT-2 toxin and ZEA could be monitored using LC-MS/MS with a "dilute and shoot" strategy employed to measure the suite of metabolites and conjugates which might be associated with irreversible bacterial metabolism (Sulyok *et al.*, 2007). Furthermore, a range of TMR components including; whole crop maize (the most important cattle fodder globally (Dunière *et al.*, 2013)) and bioethanol co-product; which is hypothesised to contain a larger relative proportion of MTs to the starting grain, especially since inferior grain may be channelled to this process by default (European-Commission, 2006b, Gallagher, 2008). Further work would be required to determine the distribution of the MTs within in the grain and whether only a proportion of present MTs are able to be degraded due to surface distribution or compartmentalisation, for it is well known that different flour milling fractions in wheat contain different concentrations of DON (Cheli *et al.*, 2013b). An observation in the current study was the absence of D3G in the silo samples, yet D3G was present in small quantities in the freshly harvested grain for both wheat and barley. It is possible that the process of ensiling releases more DON from the grain than during normal extraction and the IAC is proportionally overwhelmed by DON. This would also explain why extra dilution was necessary for IAC purification. Furthermore, the microbial assemblage and

plant enzymes present between harvesting and gamma irradiation, and the irradiation itself could break the glucosidic bond prior to the experimental incubation. *L. plantarum* has previously been found to effectively cleave D3G under laboratory conditions by Berthiller and co-workers (2011), which could help explain this anomaly.

Whilst in terms of proof of concept the inoculation of non-LAB into silage may yield a reduction in DON, however this could be at the expense of fodder quality. The Nobel prize winning pioneering research on pH and fodder preservation by Virtanen (1945) states that providing the material has a pH of between 3-4 and is free of oxygen there will be minimal losses of protein, vitamins and carbohydrate and the fodder will retain the same nutritive qualities as the starting material. This was the basis of his AIV fodder preservation process which involved the addition of mineral acids to harvested material to swiftly reduce the pH (this is the early version of Crimpstore® 2000s formulated by Kemira AIV, Finland), yielding year-round, high quality feed provision. LAB fermentation can reach the same pH threshold, but at the cost of a lag phase where a larger proportion of DM is lost. The addition of a LAB inoculant able to degrade DON whilst reducing the pH and not producing any anti-nutritional factors would be a preferable approach, hence why *W. cibaria* would be a better candidate for further testing compared to *H. alvei,* which only reduced the pH to 5.4-5.6 from 6 over 4 wk and *W. cibaria* which produced a pH of 4.3, only slightly below that of *L. buchneri* (4.1)*.* The levels of acetic acid are also similar in wheat, which is fruitful as acetic acid production in silage is usually a decent indicator of aerobic stability on silo opening, hence why *L. buchneri*-based silage additives work so effectively, in crimped grains and whole-crop maize (Driehuis *et al.*, 1999).

Whilst there is a paucity of research on crimped barley and wheat silage in the literature, there is more information on whole-crop maize silage due to its greater utilisation in global agriculture (Driehuis *et al.*, 2008). Maize is actually a promising subject for remediation of DON and *Fusarium* MTs. Maize silage has similar moisture properties, WSC content and microflora to CG whilst being more susceptible to *Fusarium* sp. infection yielding a DON content that can exceed that found in wheat. Research focussing on MT level modulation in maize has yielded some interesting results, with levels of DON reduced by almost 100% in low DM silage over 3 months (Boudra and Morgavi, 2008). The role of DM content, silage compression, use of additives and temperature in the biochemical reduction of MTs were discussed as important factors. The same authors attempted to isolate epiphytic LAB capable of DON transformation, but without success, although many of the LAB could reversibly bind *Fusarium* MTs (Niderkorn *et al.*, 2007, Niderkorn *et al.*, 2006).

This chapter is a culmination of the preceding chapters with an ongoing theme being the irreversible removal of DON using bacteria in fermented feed. Some research groups have attempted to isolate such a bacteria previously (Karlovsky, 2011), although the ideal candidate remains elusive despite some promising results presented here. Should a bacteria species be found which is confirmed to offer robust biodegradation of DON under fermented feed conditions; it will be considered a valuable addition to the armoury in the relentless battle against the debilitating effects caused by DON in feed.

6 Interactive toxicological effects of deoxynivalenol and retrorsine, two potentially co-occurring cereal contaminants, on an established human liver cell line (HepG2),

6.1 Aims and objectives

Explore the possibility that DON may contribute to the toxicity of co-occurring food and feed-borne genotoxins such as retrorsine; a pyrrolizidine alkaloid produced by *Senecio* sp. Furthermore, evaluate the mechanism by which an interactive relationship could develop using a suite of different assays to characterise the interaction in terms of organelle and biomolecule susceptibility.

6.2 Introduction

Fusarium culmorum has a particularly wide distribution in the temperate cereal growing regions of the world (McMullen *et al.*, 1997). However, it must be considered that DON is just one of many natural toxins that co-contaminate the same harvest commodity, with many other trichothecene mycotoxins derived from *Fusarium* likely to be present, such as: D3G, 3AcDON, 15AcDON and zearalenone, which contribute to the overall burden of toxicity mixtures for the consumer and health, welfare and productivity issues for livestock (Speijers and Speijers, 2004). The toxicity profile of these *Fusarium* toxins is broadly similar to that of DON and multi-residue testing strategies have been developed which could enable future prevention of dietary exposures to MT mixtures, although current safety legislation only considers each MT separately (Berthiller *et al.*, 2007). Other natural toxins identified using such methods include those derived from *Ergoflavus* sp., *Aspergillus* sp. and the plants of the group Asteraecea (Ates *et al.*, 2014). The toxins derived from these plants belong to the large pyrrolizidine alkaloid (PA) group and include retrorsine (RET) derived from ragwort (*Senecio jacobea*) and other *Senecio* sp. throughout

the globe where they are considered a serious food/feed contaminant and a cause of hepatic veno-occlusive disease (VOD) in humans with possible carcinogenic capacity (Prakash *et al.*, 1999b). RET has been trialled extensively and successfully in liver failure transplantation studies where endogenous non-hepatocyte cells are mitotically inhibited by the application of RET to prevent them outgrowing the implanted hepatocytes (Okita, 2004). The toxicity profiles of RET and DON, independently and in combination in a human liver cell line forms the basis of the current investigation.

A large amount of recent toxicological research has focused on DON and associated trichothecene MTs exposed independently or as co-existing mixtures (Wan *et al.*, 2013, Alassane-Kpembi *et al.*, 2013, Ficheux *et al.*, 2012, Ruiz *et al.*, 2011a, Ruiz *et al.*, 2011b), yet there is a paucity of data on co-occurrence and co-exposure of trichothecene MT and PAs leading to interactive toxicity. The cytotoxicity of DON is well characterised; it inhibits cellular protein synthesis at the translation stage leading to a ribotoxic stress response and apoptosis in the liver (Pestka, 2007) and impaired intestinal membrane function (Akbari *et al.*, 2014) including a compromised ability for cellular uptake of important nutrients in intestinal cells (Maresca *et al.*, 2002). Conversely, the induction of DNA damage (genotoxicity) by DON is less understood with conflicting data in the literature. For example; *in vitro* HepG2 exposures of 3.75-60 µM DON were found to produce DNA damage associated with an increased ROS production, as confirmed using co-incubation of antioxidants in the alkaline comet assay (Zhang *et al.*, 2009). Bony *et al.* (2006) found realistic and very low dietary concentrations of DON (0.01-0.5 µM) to yield significant increases in DNA damage in Caco-2 intestinal epithelial cells over 72 h (also measured using the alkaline comet assay), especially those undergoing proliferation

rather than fully differentiated cells. The same authors confirmed these doses were below the threshold level to induce apoptosis and cytotoxicity using nuclear staining and colorimetric assays. DON was conversely found to be negative as an *in vitro* genotoxin in microbial mutagenesis, with and without S9 metabolic enhancement, by Takakura and co-workers (2014). The same authors only found an increase in micronucleus formation, *i.e.* chromosome breakage/loss, when the cytotoxicity reached 55% in human lymphoblastoid cells (TK6) and speculated that at such a dose the relevance of genotoxicity is questionable as micronuclei can be artefactually formed during karyolysis following apoptosis induction. Likewise, HepaRG (human hepatoma) cells exposed to 5-35 µM DON produced an increase in apoptosis rather than genotoxicity.

RET also exerts its damage primarily in the liver due to the presence of cytochrome P450 enzymes produced by hepatocytes which metabolise RET to a reactive ester pyrrole intermediate, which crosslinks DNA and proteins causing genomic instability, megalocytosis and resultant VOD (Prakash *et al.*, 1999b, Hincks *et al.*, 1991). Wang and co-authors (2005) concluded that there was adequate *in vivo* data in rodents to suggest that RET could be a human carcinogen. Mutation spectra studies with the related PA riddelliine in transgenic rats, highlight the probable genotoxic carcinogenic mechanism of these PA as being gene transversion and tandem base substitution mutations caused by pyrrole adducts attaching preferentially to G-C base pairs (Mei *et al.*, 2004). Less subtle inter-, intrastrand DNA and protein-DNA crosslinks caused by bifunctional pyrrole adducts following metabolic activation of RET can be attributed to the megalocytosis/VOD phenomenon and cellular senescence observed by several groups (Hincks *et al.*, 1991, Fu *et al.*, 2002), in addition to mitotic and chromosomal aberrations as recorded in the

micronucleus assay. Micronucleus formation, a proxy for clastogenecity and aneuploidy, was found to be increased significantly following a 24 h exposure to 5-50 µM RET in HepG2 cells without metabolic enhancement (Kevekordes *et al.*, 2001).

To warrant an investigation on interactive toxicology it is essential that the toxins of interest (or related structural homologues) should exhibit a degree of co-occurrence in the field or during storage which results in co-ingestion and cellular co-exposure. Whilst the co-occurrence of FHB and *Senecio* sp. in the same harvest is not recorded as being a current issue in modern European agriculture, there are regions of the globe that rely on subsistence farming where weed/fungi control and grain sorting is insufficient to prevent human/animal toxicosis (Prakash *et al.*, 1999b). Furthermore, the components of compound feeds/silage/human food products can come from several harvested commodities from diverse agricultural regions that are affected by climate change and non-native plant incursions. In South Africa there have been reports of DON issues in maize (Marasas *et al.*, 1979), geographically close to areas rampant with the several toxigenic *Senecio* sp. (Dimande *et al.*, 2007). The temporal overlap between cellular exposures for each toxin needs to be such that the resulting measured toxicity can be directly linked to an interactive relationship. The simplest approach with this consideration, and the one employed in the current study, is to incubate both toxins simultaneously in a multifactorial *in vitro* dosing strategy. This constitutes an acute cellular exposure which enables putative identification of toxicity mechanisms over short temporal scales, however the toxicokinetic behaviour of each toxin within the cell and the time needed for excretion, up-regulation of enzymatic detoxification genes and induction of damage mean the results presented are a snapshot in time, and not the definitive

response characterisation. To compensate for this, a bank of several different cellular genotoxicity and cytotoxicity assays as was employed in a stepwise fashion using a metabolically competent human liver cell line (HepG2), with the results interrogated as a whole in an attempt to establish the nature of the toxicological interaction. A logarithmic dose range of at least 4 different concentrations of each toxin is tested to give an impression of dose dependant responses whilst reducing the number of samples to a manageable level. To help validate the assays for specific toxicological modes of action and help explain the responses recorded with RET/DON, two positive controls found widely in the literature are incorporated into each experiment; mitomycin-c (MMC) as a potent bifunctional, alkylating-crosslinking agent to verify concordance of the assays and aphidicolin (APH) to act as a polymerase A inhibitor to both increase sensitivity of genotoxicity assays by accumulating unrepaired damage (Speit *et al.*, 2004) and to act as a comparative co-carcinogen control.

Food and feed-borne toxicosis is a real threat to humans and production animals worldwide. Preventative measures can only be adopted following the setting out of toxicological risk. DON and RET have been shown to be toxic in a range of toxicological models when tested separately and EU food/feed safety legislation supports this, although since MTs/toxins rarely exist in isolation in food/feed this approach may be questionable (Grenier and Oswald, 2011). This study evaluates whether this is a valid approach with binary exposures of DON and RET and will attempt to establish if an interactive toxicity model is more suitable.

6.3 Material and methods

6.3.1 Cell culture and toxin treatment strategy

HepG2 cells (Human Caucasian hepatocyte carcinoma, epithelial adherent) were purchased from the European Collection of Cell Cultures (ECCAC, 85011430, Lot number 11C013, passage 101) and were cultured in MEM medium (Minimal Eagles medium, 10% foetal bovine serum (FBS), 1% non-essential amino acids, 4 mM L-glutamine, without phenol red, Gibco, Invitrogen UK) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were grown in 25 cm^2 flasks (Iwaki, Japan), sub-cultured every 6 d and re-fed every 3 d. Sub-culturing was achieved by rinsing the 90% confluent cells thrice with DPBS (Dulbecco's A phosphate buffered saline), pouring off the excess and adding 1 ml of 0.25% trypsin for 10 min until the cells were detached when 5 ml of MEM medium was added and the cells re-suspended with gentle pipetting and counted on a haemocytometer (Neubauer™). The cell suspension was diluted to 1 x 10^5 cells/ml and 5 ml added to a new flask. Triplicate experiments were carried out on passage 2-10 and cultures were handled at ACDP Category 2 containment.

All toxins were of ≥99% purity except RET (≥90%) and purchased from Sigma (Poole, UK). DON, APH and hydroxyurea (HU) were dissolved in molecular grade H_2O (Fisher), RET and MMC in cell culture grade DMSO. Log_{10} serial dilutions were undertaken in respective solvents to yield x1000 working stocks which were filter sterilised (0.22 µm) and solvents were used for controls. In each experimental well 2 µl of medium was removed for each 1000 µl medium and replaced with 1 µl of each toxin in a multifactorial combination strategy.

6.3.2 Comet assay

The single cell gel electrophoresis or comet assay is a widely used in genotoxicology where endpoints include damage to the DNA induced by xenobiotics or reactive oxygen species leading to oxidation of bases and nucleotide instability. The consequent enzymatic removal of the damaged entity or direct strand breakage leaves a gap that on separation of the sister strands at high pH, facilitates electrophoretic migration of shortened fragments of DNA visible as a cloud extending from the spherical nucleoid. Different pH conditions before and during electrophoresis yield changes in the DNA structure which allow customisation of the assay for elucidation of different damage. The pH 10 comet yields double stranded DNA fragments caused by certain crosslinking agents such as mitomycin-c (MMC) which require excision of complementary nucleotides leaving a transitory double strand break, whereas the pH 13 comet assay is more sensitive for a lower severity of damage such as oxidised bases.

In preparation of employing the technique for DON and RET treatments the assay was optimised and the measurable parameters validated at both pH 13 and pH 10 using MMC, a positive crosslinker yielding interstrand and intrastrand links, and hydrogen peroxide (H_2O_2), a positive reactive oxygen species agent producing free radical damage to DNA. In addition, APH, a nucleotide excision repair inhibitor was used as a concurrent positive control and to facilitate the accumulation of measurable DNA damage.

The comet assay was undertaken according to Tice and Vasquez (1999). HepG2 cells were seeded at 10^5 cells/ml in 12 well plates (IWAKI, Japan) and the medium changed (1 ml) 24 h later followed by addition of toxins as 1 µl volumes. Cells were incubated for 48 h, thrice rinsed with DPBS and washed off the base of the well with repeated pipetting

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(trypsin increases measurable DNA damage). Cells were centrifuged at 1000g for 3 min and the pellet re-suspended in 500 µl 0.75% low melting point agar (Sigma) at 37 °C prior to 85 µl added to a pre-coated glass slide (covered with 1% normal melting point agar in DPBS and air-dried) to make one of two gels topped with a 22 mm x 22 mm glass coverslip and chilled at 4 °C for 15 min. Both coverslips were removed and the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1% Triton X-100, 10% DMSO, pH10 with 10 M NaOH) for 24 h in the dark at 4 °C. Slides were then positioned lengthways in an electrophoresis chamber just submerged in either pH 10 or pH 13 (300 mM NaOH, 1 mM EDTA) running buffer for 40 min unwinding time plus 20 min running time at 25 V/300 mA/0.74 V/cm in the dark. Slides were removed to neutralisation buffer (0.4 M Tris, pH 7.5) for 15 min and dehydrated with ice cold MeOH and stored at room temperature until scoring. Gels were stained with 20 µl of ethidium bromide (2 µg/ml in water) and scored using a Leica-R epifluorescence microscope and x20 objective linked to a CCD camera with Komet 4 software (Kinetic Imaging, Liverpool, UK) with a 540 nm excitation/605 nm emission filter cube. Slides were blind scored and 50 cells randomly analysed for each of 2 gels per slide and the data pooled. A general linear model (GLM, Minitab 16) following square root transformation (to achieve normality) was employed for multifactorial experiments.

6.3.3 Cytokinesis-blocked micronucleus assay

Whereas the comet assay shows chemically induced DNA damage that may or may not be repaired, the cytokinesis-blocked micronucleus (CBMN) assay gives the proportion of cells with induced damage that escapes repair prior to mitosis and hence can be responsible for permanent cellular mutagenesis leading to carcinogenesis.

Such genotoxicity is scored by the presence of micronuclei which are fragments of DNA that are lost during mitosis and not incorporated into daughter nuclei, the mechanisms of loss being either clastogenic when an induced double strand break is not repaired prior to mitosis leading to loss of chromosomal material or aneugenic, when a mitotic spindle malfunction or damaged kinetochore leads to the loss of a whole chromosome. Both are catastrophic as far as cellular survival is concerned and apoptosis is the most desirable outcome. In order for the assay to confirm such irreversible damage is occurring it is necessary for cells to be actively dividing following exposure to allow irreversible loss of genetic material. This is achieved by allowing mitosis to complete, then inhibiting cytokinesis with cytochalasin-B leaving a binucleated cell. If a micronucleus or nucleoplasmic bridge is present it is likely to have been induced by exposure to the chemical treatment during the previous cell cycle. The ratio of mononucleated cells to binucleated, trinucleated and tetranucleated cells in addition to the number of apoptotic cells gives the nucleoplasmic division index (NDI), another toxicological parameter.

The MN and NDI assays were carried out according to Vevers and Jha (2008) with minor changes as described below. Cells were seeded in 12 well plates at 1 x 10^5 cells/ml for 24 h (1 ml medium/well), rinsed with DPBS and medium renewed (1000 µl) before addition of toxins. Plates were briefly mixed by hand and incubated for 48 h. Wells were then rinsed thrice with DPBS and medium added with 10 µM cytochalasin-B (in DMSO) inclusion for 24 h to inhibit actin formation and thus cytokinesis following mitosis. Cells were then rinsed with DPBS, trypsinised (0.5%), re-suspended in 250 µl freezing medium (10% glycerol in 0.075% KCl) for 10 min and stored at -80 °C in an insulated box. Scoring of cells was achieved by taking a single tube, thawing rapidly and removing 25 µl to a

glass slide followed by addition of 25 μl staining solution (10 μg/ml ethidium bromide in PBS with 0.05% Triton-X) and a 20 mm x 50 mm coverslip. Cells were viewed as the comet assay with 1000X magnification and under oil immersion (Leica-R). 1000 binucleated cells were scored from each treatment in addition to all mononucleated, trinucleated, tetranucleated and apoptotic cells observed in the process for the compiling of a nuclear division index (NDI). Aberrations recorded include those mono-, bi-, tri- and tetranuclated cells exhibiting one, two or more micronuclei, nucleoplasmic bridges, nuclear budding, mitotic catastrophe or apoptotic bodies based on the scoring criteria of Fenech *et al.* (2003), but also adopting the MN cytome approach (Fenech, 2006). The binucleated cell data is used for the main analysis as that represents the cells that have undertaken mitosis following toxin exposure. An online Fisher exact test (http://www.graphpad.com-/quickcalcs/contingency1) was employed to determine significant ($p<0.001$) increases in micronucleus induction over the control for individual treatments and a GLM was used for multifactorial experiments.

6.3.4 Cellular vitality/viability assays

Cellular viability and vitality are highly important endpoints in regards to acute and chronic toxicological effects that include reduced integrated metabolic function and impaired ability to proliferate, both which contribute to the impaired capacity for efficient working of the tissue and thus the organism as a whole. There is a balance between cellular stress and the ability of the cell to limit the effects with provision of repair mechanisms and use of alternative biochemical pathways. When the severity of the damage is beyond rescue the cell instigates the process of apoptosis, or controlled cell death, where cellular components are put out of action and mitosis halted to prevent

oncogenesis. A suite of several assays which encompass toxicological effects spanning mitochondrial function (MTT), cellular proliferation (NDI) and membrane integrity (trypan blue) were employed in the current study. When dealing with genotoxicity data it is imperative that cellular viability assays are also carried out to determine whether clastogenic and aneugenic endpoints are linked to the process of apoptosis and whether genotoxicity is only evident when the dose causes cell death and is therefore less potent in regard to carcinogenesis. The assays utilised in the current investigation for determination of different facets of cellular viability/vitality are listed in Table 6.1.

Table 6. 1. A brief description of the assays used in this investigation.

Assay	Measurable endpoints	Procedure
Comet	DNA crosslinking/strand breaks/alkali labile sites	Exposed single cells are embedded in agarose, treated with lysis solution, electrophoresed and the degree of DNA fragment migration is measured with image analysis software.
Micronucleus	Mitotic aberrations, nucleoplasmic aberrations, chromosome loss and chromosome fragment loss	Adhered cells are treated for one cell cycle with the test compound, treated with cytochalasin-B to prevent cytokinesis for 1 cell cycle and the binucleated cells scored for presence of micronuclei and other mitotic aberrations as indicative of aneugenicity and clastogenecity.
NDI	Rate of cell proliferation	The ratio of mononucleated cells to binuc-, tri- and tetranucleated cells following the cytochalasin-B treatment in the micronucleus assay is indicative of mitotic cycle effects.
Lactate dehydrogenase (LDH) leakage	LDH assayed in spent cell culture medium is a proxy for membrane leakage	Following 48 h exposure to toxins the exposate is incubated with sodium pyruvate whilst the amount of NADH converted to NAD (coenzyme, redox reaction) is measured spectrophotometrically.
MTT	Mitochondrial vitality	Toxin treated cells incubated with MTT solution for 2 h, solubilised with DMSO and amount of tetrazolium salt (purple) produced is correlated to mitochondrial activity.
Carboxyfluoroscein-diacetate metabolism/ propidium iodide uptake	Mitochondrial metabolism and cellular membrane integrity	Cells are incubated with carboxyfluoroscein diacetate/ethidium bromide for 5 min and when analysed with fluorescent microscopy/flow cytometry viable cells are green and red cells are membrane compromised.
48 h cell density (mm^2)	Rate of cell proliferation	Set up as MTT assay and after 48 h each well was visualised with an inverted microscope and cells counted in a set area.
Trypan blue exclusion	Loss of membrane integrity	As above but with addition of a dye which only penetrates compromised outer membranes so

		non-viable cells appear blue and viable cells clear.
Total cellular protein	Rate of cell proliferation	Setup as above, and after 48 h the cells were rinsed thrice with PBS and the total cellular protein content of each well determined using a BCA kit.
Shot-gun proteomics	Regulation of protein group enrichment	Extracted protein purified, digested with trypsin to peptides which are analysed with mass spectrometry to give protein group identifications.
Crosslinking assay	Determination of DNA-DNA and DNA-protein crosslinks	Extracted DNA purified, denatured at 95 °C and analysed using both spectrophotometry and gel electrophoresis to determine if crosslinking scaffolds are present to enable a swift return to double stranded DNA on cooling.

6.3.4.1 MTT assay

The MTT assay was carried out according to Cetin and Bullerman (2005). HepG2 cells were seeded in 96 well plates (Iwaki, Japan) at 2×10^4 cells/ml (200 μl) and incubated for 48 h (MEM without phenol red, 10% foetal bovine serum, 1% non-essential amino acids, 4 mM L-glutamine (all Gibco, UK), 37 °C, 5% CO_2). Cells were rinsed with PBS (Dulbecco's) and serially diluted treatments added for 48 h. 40 μl of MTT solution (5 mg/ml, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma, UK) was then added for a further 2 h in the dark (37 °C). The exposate was completely removed and retained whilst 150 μl of DMSO was added to the adhered monolayer and mixed thoroughly for the metabolised purple tetrazolium salt to solubilise. The retained exposate was then added, the plate left to settle for 5 min, 200 μl transferred to a new 96 well plate and the absorbance at 560 nm recorded. The absorbance for each treatment was normalised as a percentage of control drawn from triplicate wells in triplicate experiments. An additional experiment with D3G, DON and patulin was undertaken for validation purposes.

6.3.4.2 Lactate dehydrogenase leakage assay

Determination of extracellular membrane leakage of lactate dehydrogenase (LDH) in treated cells was carried out using a protocol devised by Bergmeyer and Bernt (1974).

For each sample 50 µl of cell-free supernatant (CFS) was added to one well of a 96 well microtitre plate and 250 µl assay mix was added (180.9 µM NADH, 4.55 mM sodium pyruvate in 10 ml 50 mM potassium phosphate buffer pH 7.5). The decrease in absorbance at 340 nm was monitored on a Spectromax plate reader every 11 s for 30 min and represents the redox conversion of the coenzyme NADH to NAD$^+$. The initial gradient of absorbance decrease was used for comparison between treatments and compared with a serial diluted LDH activity unit standard (~140 U/mg, Sigma).

6.3.4.3 Carboxyfluorescein diacetate metabolism assay

This fluorescence based live/dead assay was undertaken according to Tice and Vasquez (1999). Briefly, HepG2 cells were treated with toxins for 48 h, rinsed with PBS and trypsinised (0.05%) and re-suspended with MEM medium with FBS. The cells (2 x 10^5) were centrifuged at 1000g for 2 min and the supernatant replaced with 10 µl of carboxyfluoroscein diacetate (CFDA): propidium iodide working solution (62 µg 5-6,-carboxyfluorescein diacetate, 12 µg propidium iodide in 1 ml DPBS) for 5 min at 37 °C. The pellet was rinsed twice with DPBS, re-suspended in 1 ml DPBS in FACS tubes and analysed within 1 h using a flowcytometer (excitation 520 nm/emission 620 nm, FACS Aria, Becton Dickinson, San Jose, USA). The acetate is cleaved by cytosolic esterases in viable cells leaving a fluorescent product. 10,000 events were characterised with green fluorescence an indication of cellular viability and red fluorescence an indication of compromised membranes and low viability. The same population of cells was dropped on a microscope slide for manual scoring and saving images using a Nikon epifluorescence microscope using BR Elements image analysis (Nikon). 200 cells were scored to confirm flowcytometric gating and analysis.

6.3.4.4 Determination of apoptosis by annexin V-FITC staining

Apoptosis was determined using a kit from eBioscience Inc. (UK) following the supplied protocol as follows. Exposed cells were trypsinised (as described previously), pelleted and rinsed with DPBS, binding buffer, then re-suspended at 1×10^6 / ml prior to mixing 100 µl of this cell suspension with 2.5 µl of fluorescein isothiocyanate (FITC)-conjugated Annexin V for 15 min at room temperature. Cells were washed in binding buffer, and re-suspended in 300 µl binding buffer, counter stained with 7.5 µl of propidium iodide solution (as previously used) and analysed both with flowcytometry (FACS aria, set up as previously) and fluorescent microscopy (as described previously). Fluorescent annexin V conjugates bind to phosphatidylserine which is presented on the surface of apoptotic cells but can however attach to phosphatidylserine on the cytoplasmic surface of cells with compromised membranes and therefore the membrane impermeable propidium iodide is also added to distinguish necrotic cells from apoptotic cells.

6.3.4.5 Trypan blue and monolayer cell density

Cells were treated as per the MTT assay, the cells rinsed with DPBS and the supernatant removed and replaced with 20 µl 0.4% trypan blue stain (Gibco) for 20 min prior to counting at x40 on an inverted microscope (Nikon, UK) with an eyepiece graticle counting grid in the eyepiece. Both viable (clear) and non-viable membrane compromised (blue) cells were recorded. A 2 mm stage micrometer was used to calculate the area highlighted by the eyepiece graticule (1 mm^2). Microscopic examination of cell numbers is exceptionally important when considering colorimetric cytotoxicity data.

6.3.4.6 Protein assay

Determination of cellular protein was undertaken using the bicinchoninic acid method (BCA kit, Pierce) using the manufacturer's instructions. Briefly, cells exposed in 96 well

plates were carefully rinsed thrice with DPBS (to prevent interference from FBS) prior to addition of 250 μl of the freshly mixed working reagent (1:50 mix of A and B) to each well and incubation at 37 °C for 45 min in the dark. The plate was gently vortexed (IKA plate shaker, 60 rpm) and 200 μl was removed to a fresh well in a new plate for absorbance reading at 562 nm and determination of protein content using a calibration plot made using 10 μl of BSA standards (0.1-2 mg/ml).

6.3.5 Proteomics

6.3.5.1 Preparation of cellular protein for proteome analysis

Proteomic preparation and analysis was undertaken according to Edwin Lasonder (pers. comm.). All reagents were LC-MS grade (Sigma) and manipulations were undertaken using strict aseptic technique to prevent human and ovine keratin contamination prior to trypsinisation. The cells from 3 replicate experimental well treatments were pooled (500 μl of each) and centrifuged at 8000 g in Lo-bind tubes (Eppendorf) for 3 min prior to addition of 500 μl RIPA (Radio-immunoprecipitation assay) buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% TritonX-100, cocktail protease inhibitor (Sigma), pH 8). The protein content was determined using a BCA kit (Pierce) and diluted to 1 mg/ml prior to 30 μl being mixed with 10 μl Biorad gel loading buffer (4X) and pipetted into a Biorad Criterion XT 10% Bis-tris gel and electrophoresed at 100 V for 2 h in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). This enabled cleaning the proteins of residual contaminants. The gel was then fixed (1% AA, 40% MeOH) for 1 h on an orbital shaker (45 rpm, SSL1, Stuart Scientific, UK). The gel was then stained with Coomassie blue (0.1%, Sigma) and then de-stained (10% AA, 25% MeOH) for several days on a laboratory orbital shaker. When only the separated bands were visible

the gel was cut with a sterile scalpel into 4 equal fractions for each lane and placed in a Lo-bind tube and centrifuged briefly. The excised gel piece was de-stained by repeating the following cycle: addition of 100 µl 50 mM ammonium bicarbonate (ABC, Sigma, LC-MS grade) for 5 min then discarding all liquid, addition of 100 µl 50% ACN and discard, followed by 100 µl of 100% ACN and discard. To achieve protein reduction the slice was incubated at 50 °C with 100 µl of 10 mM dithiothreitol in H_2O. The gel slice was then dehydrated in 100% ACN (5 min) prior to addition of 50 mM 2-chloroacetimide (in ABC, in the dark) to alkylate the proteins. To equilibrate the slices, 100 µl of 100% ACN was added, incubated for 5 min, then the same with ABC and this cycle repeated twice more. The gel slices were then immersed in enough digestion buffer (12.5 ng/µl trypsin (Thermo high fidelity) in ABC) over the course of 30 min to allow complete absorption by the gel slices plus enough to cover them for incubation at 37 °C for 16 h to digest the proteins down to peptide precursors. The tubes were then sonicated for 20 s (Fisher, UK), centrifuged briefly and 50 µl of 2% trifluoroacetic acid in H_2O (TFAA, Sigma) added prior to a 15 min incubation at 1400 rpm (Thermomixer Comfort, Eppendorf) and centrifugation to collect supernatant to a fresh tube. This was repeated with buffer B (80% ACN, 0.5% acetic acid (AA), 1% TFAA) and both supernatants pooled and concentrated down in a vacuum centrifuge (Centrivap micro IR, LabConCo, USA) at 45 °C, 1000*g* with lids off until all ACN had evaporated (by drawing a line after first amount of supernatant added) and 5 µl of 100% TFAA added to acidify the samples.   Peptides were cleaned up by passing the sample through C18 stage tips. The stage tips were constructed as follows: A sheet of C18 membrane was wetted with MeOH and 2 mm discs were cut out using a cut off 200 µl tip. A Hamilton syringe was used to push 2 discs (1 at a time) into a new 200 µl tip. This stage tip was then inserted into the lid of a new lo-bind centrifuge tube.

Stage tip activation was accomplished as follows: 50 μl of MeOH was added and centrifuged at 1000*g* for 2 min (same settings for all following steps), 50 μl of buffer B was then added and centrifuged followed by 2 cycles of 25 μl buffer A (0.5% AA, 1%TFA in H$_2$O). Sample was then added to the stage tip followed by 50 μl buffer A to remove unbound peptides. The sample was then eluted with 2 cycles of 25 μl buffer B into a fresh tube, vacuum centrifuged to a total volume of 8 μl, 25 μl of buffer A added to each tube and the contents transferred to 300 μl glass vials (Chromacol, UK).

6.3.5.2 Nano-HPLC

A Dionex Ultimate 3000 U-HPLC (Thermo Scientific, Hemel Hempstead, UK) was employed for the separation of the peptides prior to mass spectrometry. 5 μl of digest was injected using the μl pick-up facility onto a trap column (Pepmap 75 μm x 2 cm, Thermo) for 5 min (loading pump, buffer A, 1 μl/min) to remove contaminants. The valve position was then changed to include a Pepmap 100 analytical column (C18, 3 μm, 75 μm x 25 cm, Thermo) for the main peptide separation using the following gradient settings at 0.3 μl/min: 0 min 4% buffer B (80% ACN + 0.5% AA), 9.8 min 4% buffer B, 10 min 5% buffer B, 129 min 32% buffer B, 130 min 90% buffer B, 140 min 90% buffer B, 141 min 4% buffer B and 155 min 4% buffer B. The autosampler was kept at 5 °C and the column compartment at 32 °C. Two blank injections were run between 4 fractions of the same sample (4 vials).

6.3.5.3 Mass spectrometry

An LTQ Velos Pro (Thermo Scientific ,Hemel Hempstead, UK) equipped with an Orbitrap fourier transformed mass spectrometer (FTMS) was externally calibrated and the U-HPLC separate peptide sample nebulised by Nanospray ionisation (NSI, spray voltage 2.5 KV, capillary temperature 275 °C). The first scan event was achieved in the FTMS using the following settings: mass range of 350-1500 Da, resolution of 60000, full scan type,

positive polarity, CID isowidth of 1.0, normalised collision energy of 35, Act Q of 0.250, Act time of 10 ms. The second scan event (MS^2) was undertaken in the ion trap with a normal mass range and scan rate.

6.3.5.4 Analysis of mass spectrometry data with MaxQuant 5.1 and DAVID database searching

MaxQuant 5.1 (Max Planck Institute, Germany) was employed to determine total number of protein groups and the prevalence of each protein using label-free quantification (using ion intensities). An experimental design grouped the four fractions analysed in U-HPLC together for each treatment and the 16 mass spectrometry data files (4 x 4) using the following settings: modifications = oxidation and deamidation, max number of modifications per peptide (2), first search (20 ppm), second search (6 ppm), max charge (5), MS/MS tolerance of FTMS (20 ppm, 10 peaks per 100 Da), Fixed modification (Carmidomethyl (c)) with contaminants included. The FASTA files used for searching were human.first.search.fasta, IPI.Human.V3.68.FASTA and were downloaded from NCBI (USA) with search identification and quantification settings as follows: peptide FDR (0.01), side FDR (0.01), max peptide PEP (0.001), min (0), min peptide length (6).

Output data was uploaded as IPI format to DAVID, a proteomic enrichment tool (http://david.abcc.ncifcrf.gov/home.jsp), and grouped by biological functions including: protein biosynthesis, translational elongation, apoptosis, membrane function, response to toxin and cell cycle regulation. The top 100 proteins in terms of integrated intensity peak value in the control for each category was taken for comparison between treatments and displayed as cumulative intensity in order of descending prevalence. In addition, any protein with enrichment 4-fold deviation (25 / 400% of control expression)

from the control was identified and grouped into the previous functional groups if possible. An additional group of proteins of special relevance is also given.
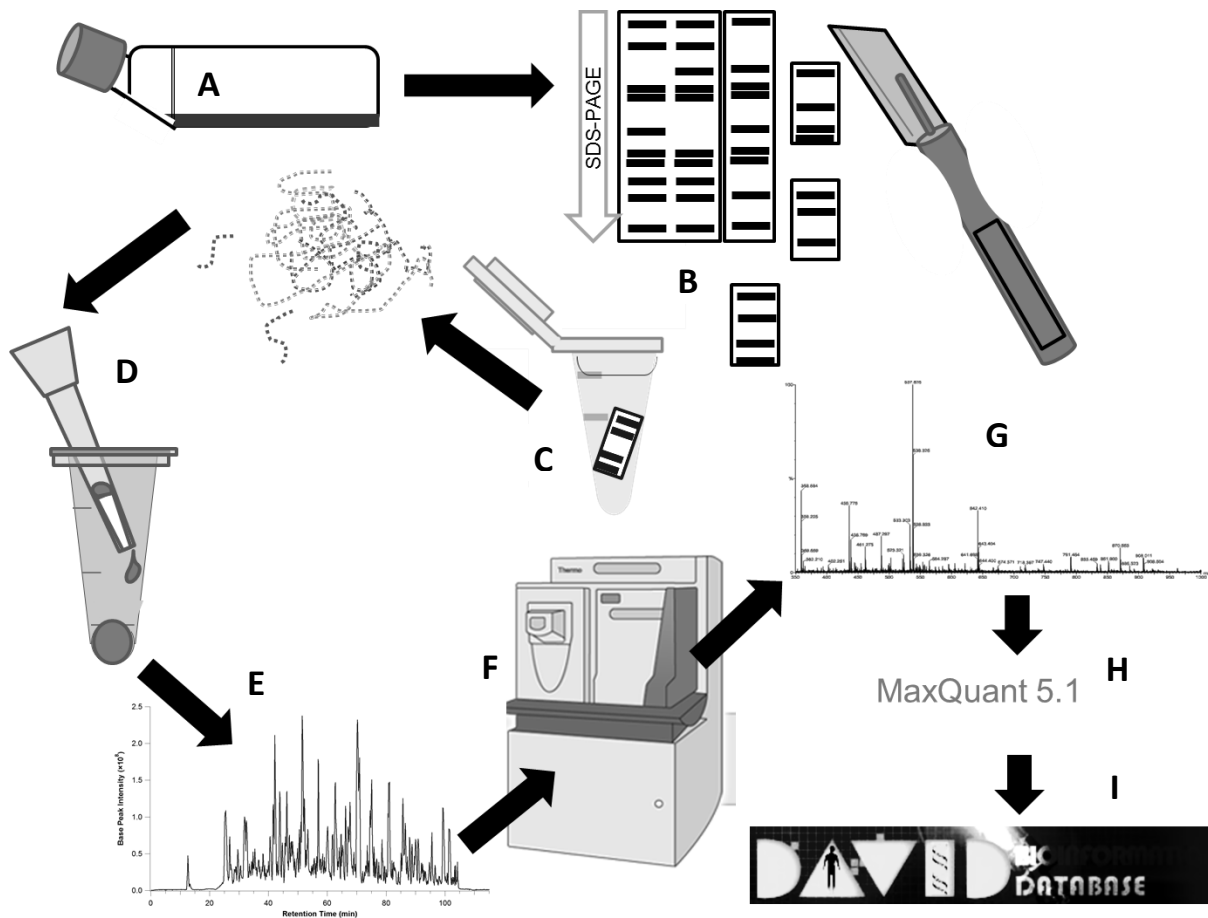
Figure 6.1. Simplified schematic diagram of sample preparation and analysis required for shot-gun proteomics. A, toxin treated HepG2 cells extracted for total protein and diluted to normalise between treatments; B, extracted proteins separated with SDS-PAGE, stained and gel slices dissected; C, gel-bound proteins incubated with trypsin to produce peptides that are extracted from the gel; D, peptides cleaned using C18 membrane stage tips; E, peptides separated by U-HPLC; F, separated peptides ionised, fragmented and analysed by Orbitrap™; G, mass spectra data of top 10 peptides for each scan event saved; H, data interrogation yielding protein group identification and label free quantification between groups with MaxQuant 5.1; I, protein group enrichment and gene functional annotation clustering analysis using DAVID™ bioinfomation database software. Velos Pro diagram and chromatograms courtesy of Thermo Scientific™.

6.3.6 Crosslinking assay

To determine the degree of crosslinking occurring following combinational exposure an absorbance based and gel based assay was devised based on earlier work by Tang *et al.* (2008b). Briefly, triplicate experimental treatments (in 25 cm^2 flasks) were pooled and DNA was extracted with a Promega™ mouse tail DNA extraction kit using the manufacturer's instructions. DNA purity and concentration was measured using a Nanodrop and the concentration normalised to 100 μg/ml by dilution with TE buffer. 25 μl of diluted sample was added to a microcentrifuge tube and subjected to denaturing at 95 °C for 10 min on a heating block (Techne, UK) and then put on ice, then 2 μl was removed and absorbance measured at 280 nm on a nanodrop spectrophotometer (time zero) and two further readings taken at 20 min intervals. An increase in absorbance indicative of strand separation and a rapid annealing suggestive of crosslinking scaffolds speeding up alignment.

A similar approach was employed for a gel-based analysis of crosslinking utilising the ability of denatured DNA to anneal more swiftly when crosslinking scaffolds are in place and are hence less likely to migrate as single stranded DNA fragments. Following denaturing the 25 μl of DNA put on ice and 8 μl was mixed with 2 μl loading buffer (X5, Bioline, UK) before pipetting into a well in a 1% agarose gel in TE buffer, staining with 0.01% SYBR safe (Invitrogen, UK) and running it at 100 V for 2 h at 4 °C under red lighting. Positive controls for denaturing consisted of DNA ladders (Easyladder I (2Kb-100bp) and II (5Kb-500bp), Bioline, UK) treating in the same way as the samples with 10 μl applied. The gel was then visualised on a GelDoc system (Applied Biosystems).

6.3.7 Statistical analysis

Statistical analysis was undertaken using Minitab 16 Statistical Software (Minitab Inc.). Unless stated separately, a general linear model (GLM) was undertaken for each assay with the interaction term made up of different concentrations of DON and RET. A post-hoc Tukey's comparison was used for defining significant differences between groups where necessary (an asterisk represents significant departure from the control, or shared letters represent those treatments do not deviate significantly, all at $P<0.05$).

6.4 Results

6.4.1 Comet assay

6.4.1.1   Validation results

When attempting to obtain reliable comet data with crosslinking and free radical agents it is imperative to develop a robust assay protocol that is dependent on cell type and type of damage anticipated. An unwinding time of 40 min and electrophoresis time of 20 min resulted in a consistent and measurable response in the controls that enabled both an increase (strand breaks) and reduction (cross-linked strands) in DNA migration to be recorded (dependant on the mode of genotoxicity). The pH of the electrophoresis buffer was a substantial factor regarding the presentation of measureable damage (Figure 6.2, 6.3). There are a wide range of measurement parameters in the comet assay which can be used to assess DNA modifications that include tail length, percentage of DNA in the tail and Olive tail moment (length and intensity). All three of these are compared with positive controls for free radical damage (H_2O_2) and crosslinking adducts (MMC) in order to find the parameter which gave the best dose response and concordance (Table 6.2).

In general pH 13 and tail length gave the greatest r-square values for both H_2O_2 and MMC treatments, in addition to the data fitting a normal distribution allowing parametric statistics to be undertaken. H_2O_2 increased tail length dose dependently at both pH10 and 13. For MMC induced crosslinking the pH 10 comet yielded opposite responses to that of pH13 in terms of correlation plots. This suggests major DNA fragmentation such as incidence of double stand breaks decreases with dose, yet there is an increase in pH labile single strand breaks at pH 13 as damage surpasses a threshold. All future comets were undertaken at pH 13 with tail length the most appropriate parameter to use for statistics. All comet data was square root transformed to achieve normality.

Table 6. 2. Comet validation data with comparison of different comet measurement parameters following exposure to MMC or H_2O_2 with comet assay conditions of pH 10 or pH 13. The suitability of each parameter is derived by comparing the degree of correlation between treatment dose and comet parameter value, the suitability of the data for ANOVA employing an Anderson-Darling test of normality and the robustness of ANOVA (R2 value, F-statistic and significance) leading to confidence in deriving significant difference between doses.

Treatment	Statistics	pH 10 Comet parameters			pH 13 Comet parameters		
		% Tail DNA	Olive tail moment	Tail length	% Tail DNA	Olive tail moment	Tail length
MMC	Correlation	0.361, <0.001	0.462, <0.001	-0.514, <0.001	-0.115, <0.001	-0.198, <0.001	0.409, <0.001
	Normality	0.998, 0.068	0.990, <0.01	0.984, <0.01	0.993, <0.01	0.963, <0.01	0.987, <0.01
	ANOVA	16.48%, F=52.34, <0.001	26.49%, F=95.64, <0.001	27.67%, F=101.49, <0.001	14.77%, F=45.99, <0.001	22.15%, F=75.50, <0.001	30.10%, F=114.28, <0.001
H_2O_2	Correlation	0.063, 0.077	0.028, 0.433	0.505, <0.001	0.513, <0.001	0.614, <0.001	0.379, <0.001
	Normality	0.999, <0.1	0.998, <0.1	0.988, <0.01	0.991, <0.01	0.978, <0.01	0.987, <0.01
	ANOVA	13.6%, F=41.76, <0.001	14.87%, F= 46.33, <0.001	33.37%, F=132.86, <0.001	46.40%, F=229.69, <0.001	55.99%, F=337.62, <0.001	43.54%, F=204.62, <0.001
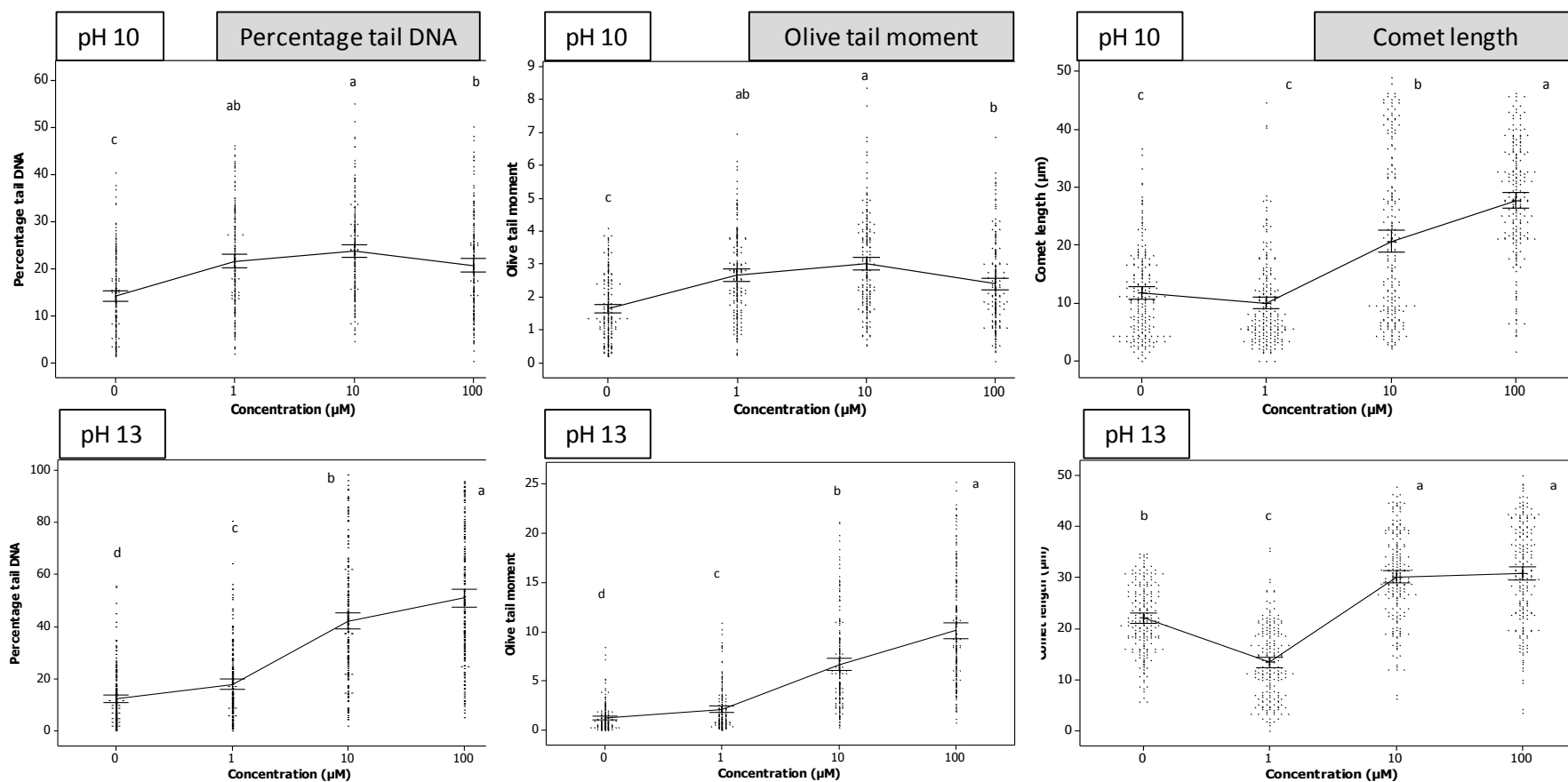
Figure 6.2. Comparison of parameters for the validation of the comet assay using DNA damage caused by exposing HepG2 cells to a range of H_2O_2 concentrations for 10 min followed by electrophoresis at pH10 or pH13 in the comet assay. The mean, standard error and pooled individual data points of triplicate experiments is shown. Treatments sharing the same letter are not significantly different (<0.001).
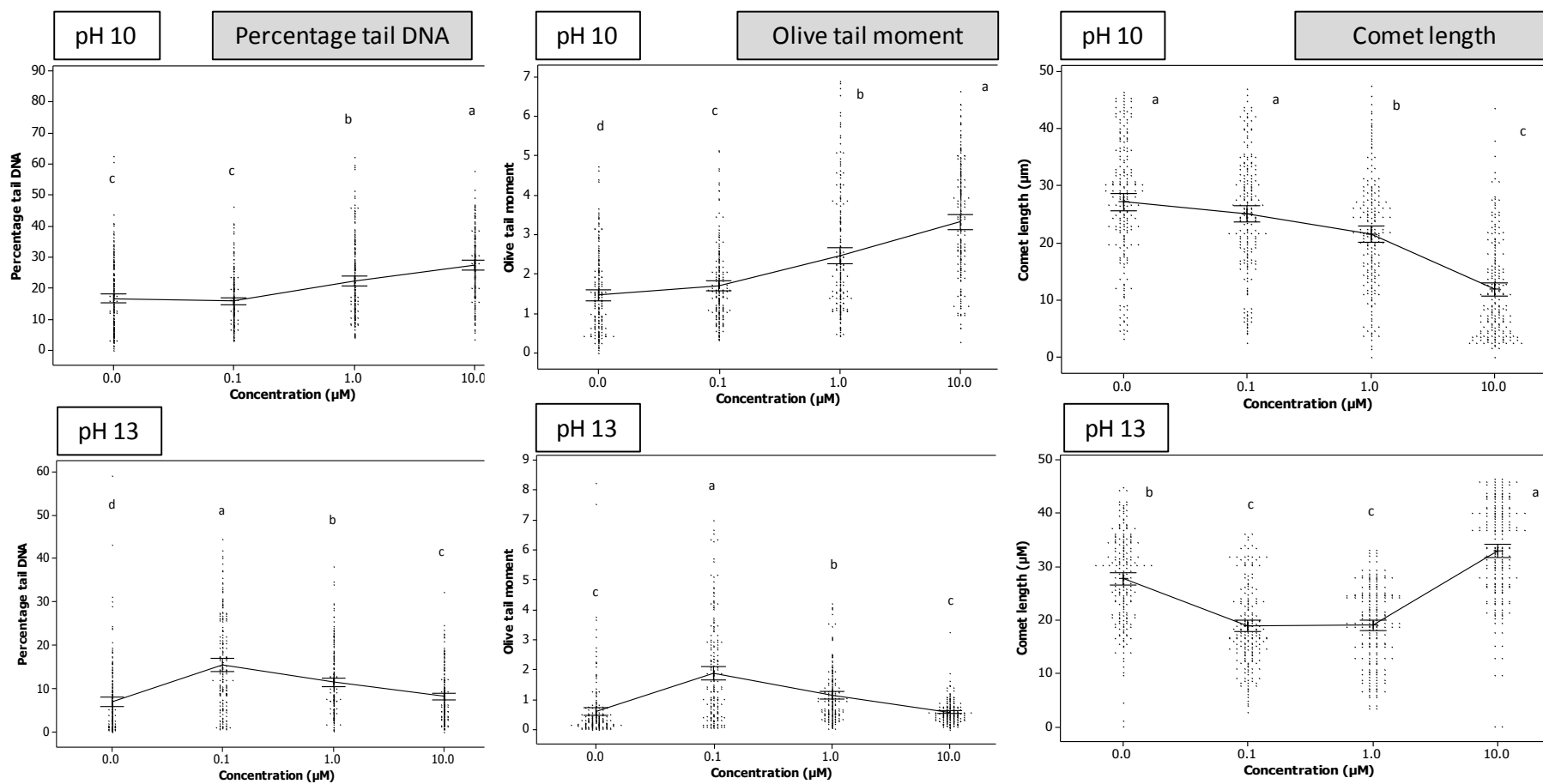
Figure 6. 3. Comparison of parameters for the validation of the comet assay using DNA damage caused by exposing HepG2 cells to a range of MMC concentrations for 40 min followed by electrophoresis at pH10 or pH13 in the comet assay. The mean, standard error and pooled individual data points of triplicate experiments is shown. Treatments sharing the same letter are not significantly different (<0.001).

6.4.1.2   Multifactorial comet experiment

The multifactorial comet assay involved exposing HepG2 cells for 48 h to binary exposures of 0, 1, 10 and 100 µM RET combined with 0, 0.1, 1 and 10 µM DON or 1.5 µM APH (Figure 6.4). The mean tail length is shown with the individual cell values for each treatment to give an idea of clusters indicative of apoptosis, chromatin condensation or stalling of the cell cycle. Figure 6.5 is provided to more clearly illustrate the differences between treatments.
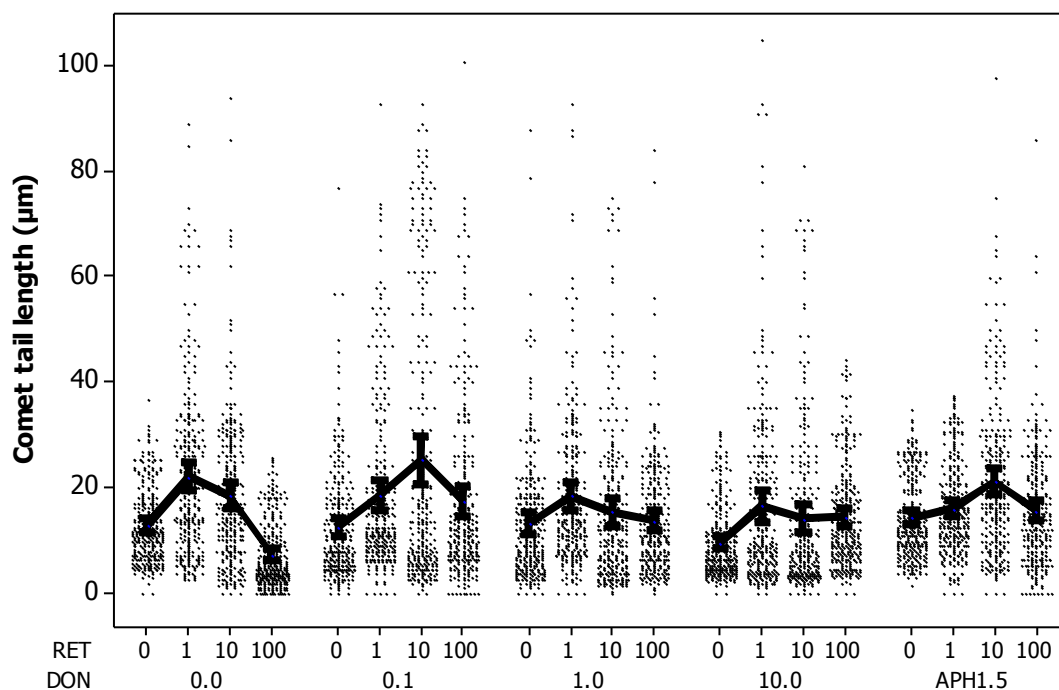
Figure 6. 4. Comet tail length as representative of DNA damage and crosslinking caused by DON and RET with APH (µM) as positive control for DNA repair inhibition. The mean is shown with the standard error in addition to individual data points pooled from 3 experiments.
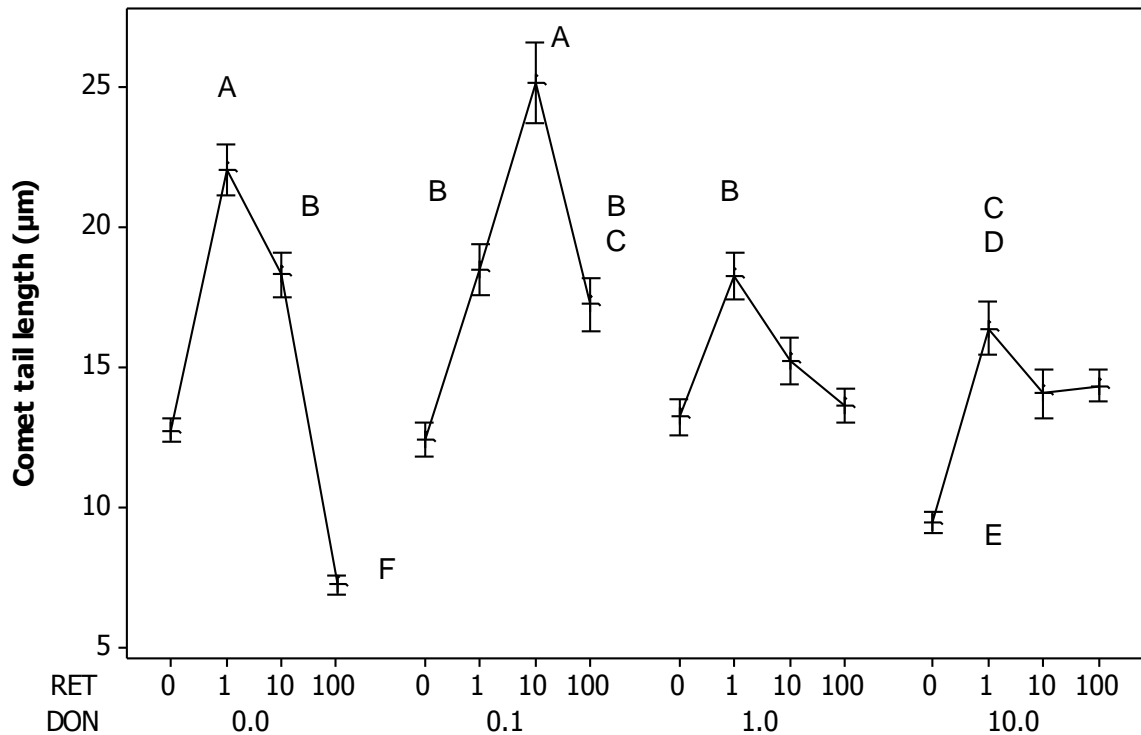
Figure 6. 5. Comet tail length as representative of DNA damage and crosslinking caused by DON and RET (µM). Data points sharing the same letter to not differ significantly (*P*<0.05). The mean is shown with the standard error from 3 experiments. Enlarged version of 6.4.

The comet assay data gives the impression that the interaction is more complex than a linear dose dependant relationship, although RET in isolation does yield a clear bi-phasic dome-like relationship. This RET-induced restriction in migration is presumably due to DNA-DNA and DNA-protein crosslinking of DNA, and is particularly evident as nucleoids with virtually non-existent migration of DNA at the highest dose (100 µM). DON in isolation does not cause any difference in DNA migration in the lowest doses and even reduces migration at the highest dose (10 µM), the opposite to the hypothesis of the higher dose yielding more apoptosis and hence, more diffuse and longer comet tails. The lack of cell division and translation of RNA may mean the DNA is compact as replication and protein synthesis is halted (presumably the histones are not being synthesised either). When we consider the interaction of RET with DON it becomes highly complex. Low concentration of DON both decreases the migration of DNA at low doses of RET

suggesting either lesions are not occurring or overwhelmed DNA-repair is not excising nucleotides to leave a gap. A high dose of DON (10 µM) with 100 µM RET gives a suggestion of crosslinking inhibition. Addition of APH yielded a similar response to DON in that both increases and decreases in migration were reduced. The plotting of individual cell migration length in addition to the mean allows better interrogation of the dose response in terms of effects on the cell cycle chromatin condensation dynamics and karyorrhexis/karyolysis during apoptosis. When comparing the mean with the actual spread of the data it is apparent that the mean can be quite misrepresentative of the outlying groups of cells falling into the apoptotic and cross-linked categories, a phenomenon perhaps more important with crosslinking mechanisms.

An increase in MMC concentration yielded a significant increase in tail length compared with the control and 0.1 µM MMC (as found in the validation experiments). This response was increased with 10 µM DON co-exposure suggesting a mechanism exists that results in non-repair of damage and possible disruption of DNA metabolism (Figure 6.6). This interaction is different to RET/DON where DON reduces migration. APH 1.5 µM increased the response found with 10 µM MMC in isolation which suggests inhibition of polymerase does result in single strand gaps following NER of cross-linked nucleotides. It also supports the theory that APH can be used to increase comet assay sensitivity. A 48 h H_2O_2 exposure produced a linear response of oxidation damage which was reduced compared with a 10 min exposure and it is apparent from the wide distribution of tail lengths that apoptosis has been initiated in the highest concentration.
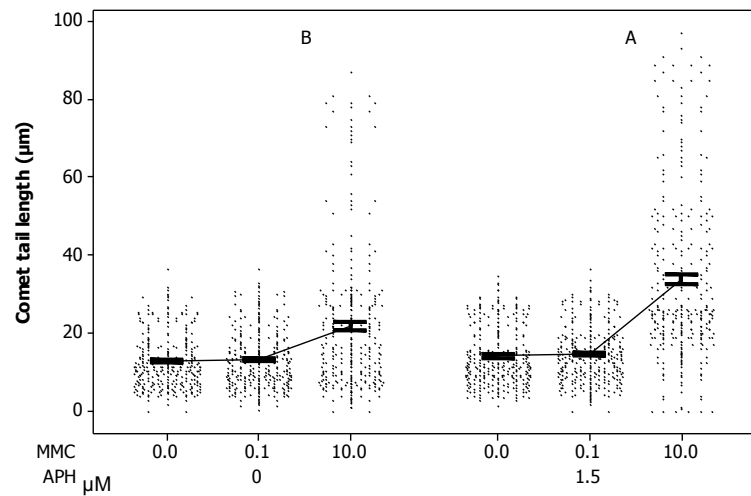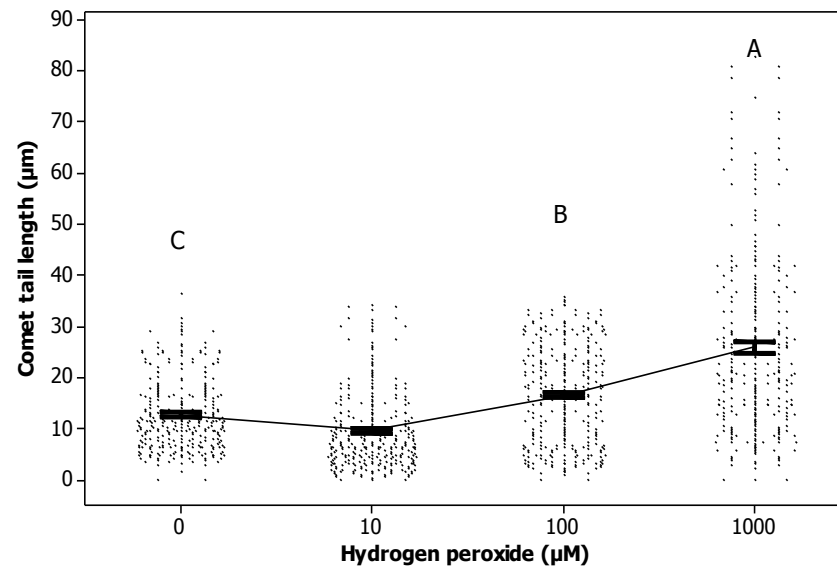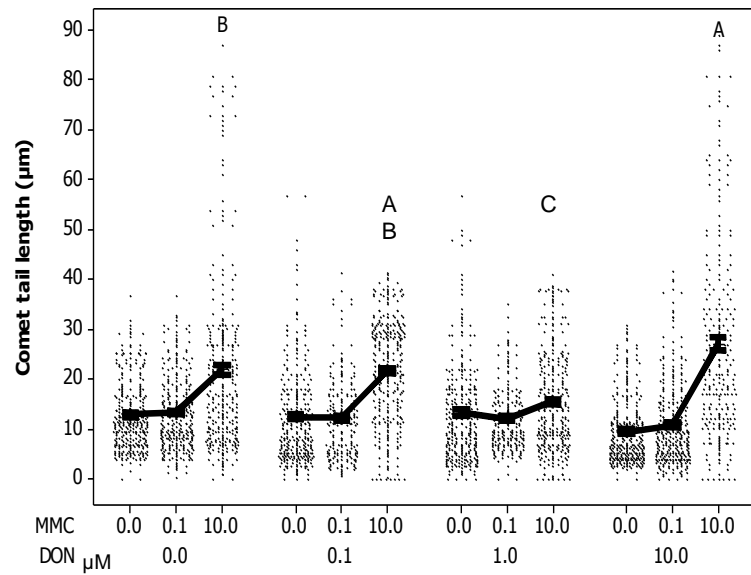
Figure 6. 6. Comet tail length as representative of DNA damage and crosslinking following a 48 h exposure of HepG2 cells to varying µM concentrations of MMC and DON (top left), MMC and APH (lower left) and H2O2 for 48 h in MEM medium (above). The mean is shown with the standard error in addition to 300 individual comet lengths (µm) pooled from 3 experiments.

6.4.2 Cytokinesis blocked micronucleus assay

6.4.2.1   Preliminary 48 h Micronucleus assay with RET and MMC
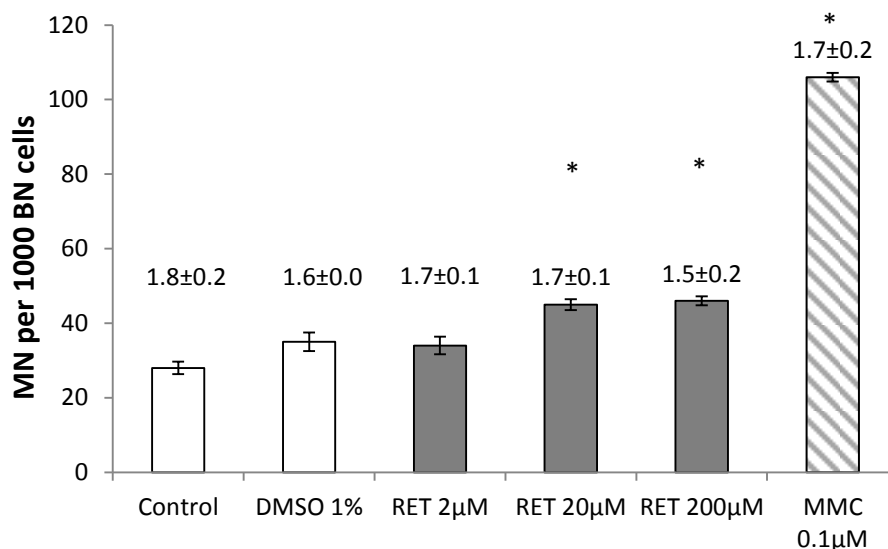
Figure 6. 7. The mean incidence of micronuclei/1000 binucleated cells is shown following 48 h exposure to RET and MMC. The nucleoplasmic cytotoxicity index and standard error of triplicate experiments is shown with asterisks denoting a significant difference to the control (*P*<0.05).

There was a significant correlation between RET dose and irreversible loss of genetic material (either whole or fragments of chromosomes) when tested with concentrations reported in the literature. The MMC dose of 0.1 μM gave an adequate positive control result. The NDI (nuclear division index) values indicate DMSO had a slight effect on cellular integrity and turnover, with this increasing with each concentration of RET. The dose of MMC used as a control has a NDI index comparable with the control indicating genotoxicity induced without excessive cytotoxicity illustrating the potential for MMC to act as a potent carcinogen and be a suitable positive control for such effects.

6.4.2.2   Preliminary 48 h Micronucleus assay with DON, APH, hydroxyurea and MMC
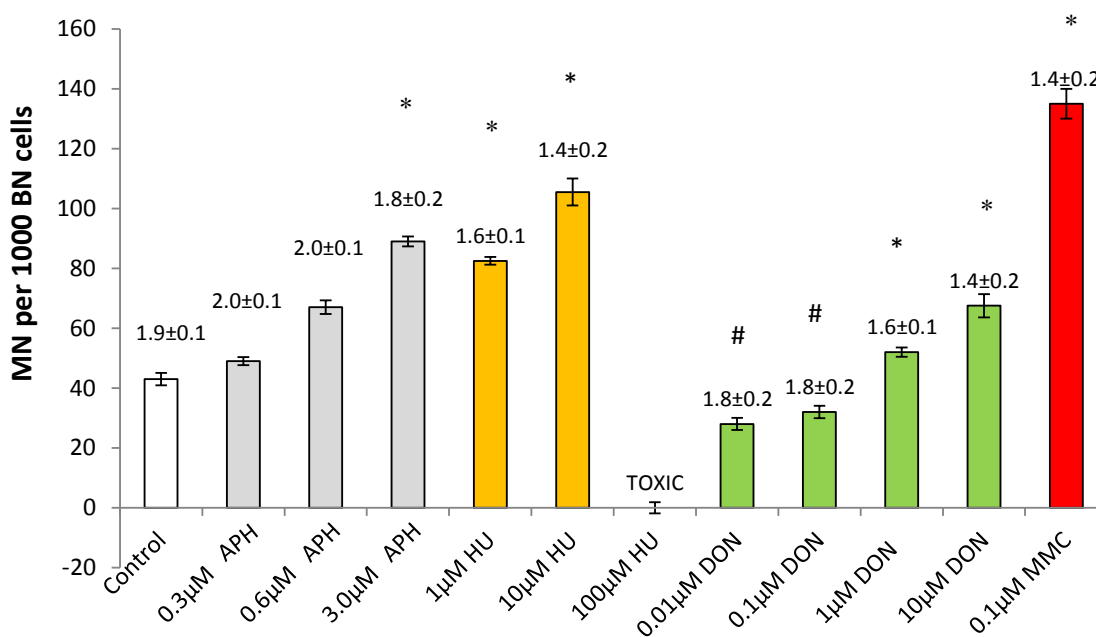
Figure 6. 8. The mean incidence of micronuclei / 1000 binucleated cells is shown following 48 h exposure to APH, hydroxyurea (HU), DON and mitomycin-c (MMC). The standard error of triplicate experiments in shown along with the nucleoplasmic cytotoxicity index. Significant micronucleus incidence above the control is shown with an asterisk and below control frequency (#).

6.4.2.3   Multifactorial micronucleus assay

Table 6.3. Mean incidence of specific aberrations scored in the micronucleus assay following 48 h treatments of DON, RET, MMC and APH. The mean counts/1000 BNC of 3 experiments is shown with the SE.

DMSO	DON 0 μM	±SE	DON 0.1 μM	±SE	DON 1 μM	±SE	DON 10 μM	±SE	DON 100 μM	±SE	APH 0.5 μM	±SE
BNC with 1 x MN	33	9	35	13	27	9	22	4	55	5	55	23
BNC with 2 x MN	0	0	3	3	10	10	17	7	10	10	15	7
BNC with 2+ x MN	5	5	0	0	13	9	10	6	15	15	24	9
BNC with bleb	12	6	19	1	3	3	3	3	15	15	13	10
BNC with bridge bleb	5	3	6	3	5	5	5	5	10	10	16	9
BNC with nucleoplasmic bridge	39	18	82	11	74	33	51	26	145	45	60	22
RET 1 μM	DON 0 μM	±SE	DON 0.1 μM	±SE	DON 1 μM	±SE	DON 10 μM	±SE	DON 100 μM	±SE	APH 0.5 μM	±SE
BNC with 1 x MN	33	9	23	3	57	21	53	34	40	30	58	4
BNC with 2 x MN	19	12	7	3	10	10	27	12	15	5	12	7
BNC with 2+ x MN	6	3	0	0	18	4	7	7	10	0	18	11
BNC with bleb	14	5	3	3	14	8	3	3	20	20	8	2
BNC with bridge bleb	14	2	3	3	15	9	17	12	30	0	15	5
BNC with nucleoplasmic bridge	55	12	110	42	78	18	107	41	145	45	50	15

RET 10 µM	DON 0 µM	±SE	DON 0.1 µM	±SE	DON 1 µM	±SE	DON 10 µM	±SE	DON 100 µM	±SE	APH 0.5 µM	±SE
BNC with 1 x MN	51	19	26	5	65	17	44	6	56	8	57	5
BNC with 2 x MN	18	6	9	6	17	1	10	6	20	4	37	14
BNC with 2+ x MN	13	7	3	3	5	5	0	0	12	4	19	7
BNC with bleb	13	13	15	9	8	8	3	3	0	0	5	5
BNC with bridge bleb	15	9	15	7	15	4	3	3	8	8	9	5
BNC with nucleoplasmic bridge	81	32	42	14	69	11	51	24	216	32	59	17
RET 100 µM	DON 0 µM	±SE	DON 0.1 µM	±SE	DON 1 µM	±SE	DON 10 µM	±SE	DON 100 µM	±SE	APH 0.5 µM	±SE
BNC with 1 x MN	32	8	30	6	27	9	57	19	42	18	69	20
BNC with 2 x MN	7	7	2	2	7	3	23	23	0	0	11	7
BNC with 2+ x MN	3	3	10	10	18	7	3	3	4	4	19	10
BNC with bleb	3	3	2	2	12	7	10	10	4	4	15	9
BNC with bridge bleb	21	6	2	2	12	7	23	23	24	16	22	4
BNC with nucleoplasmic bridge	57	12	32	13	58	14	67	9	120	40	50	6
MMC 0.1 µM	DON 0 µM	±SE	DON 0.1 µM	±SE	DON 1 µM	±SE	DON 10 µM	±SE	DON 100 µM	±SE	APH 0.5 µM	±SE
BNC with 1 x MN	53	20	83	38	57	9	47	9	TOXIC		77	26
BNC with 2 x MN	43	3	10	10	13	7	7	3			20	12
BNC with 2+ x MN	20	6	17	12	13	7	10	6			30	17
BNC with bleb	7	3	7	7	13	3	7	3			0	0
BNC with bridge bleb	3	3	7	7	3	3	27	15			7	7
BNC with nucleoplasmic bridge	37	9	93	24	100	35	87	3			73	35

Linear dose responses for APH (aphidicolin), HU (hydroxyurea) and DON were found in a further validation micronucleus experiment. The nuclear division cytotoxicity index decreases with dose indicating increased cell division with increasing doses of all treatments apart from APH. When the sum of all aberrations scored is compared (MN, bridges, blebs, bridge-blebs) between the doses of RET in isolation, the dose response shows a dome shape in common with the comet assay results. An increase in RET concentration yields a stepwise increase in number of MN and nucleoplasmic bridges from 1 to 10 µM with the top dose of 100 µM producing a reduction in the amount of recordable aberrations. When considering the combinational response these non-significant trends lead to a diminished dome-like response. GLM analysis (R-Sq = 44.2%) suggests DON yields the main effect (F = 4.08, P = 0.008), with concentration of RET (F =

1.37, P = 0.266) and interactive components (F = 0.757, P = 0.68) not contributing significantly (Figure 6.9).

With an increase in DON concentration to 100 µM this trend is heightened with the number of scorable aberrations doubling, however the middle concentrations of DON have a far more complex relationship. It is apparent that a small amount of RET (1 µM) induces a degree of damage that is modulated in a DON-dose dependant way. Due to the complex toxicological response it is impossible to describe the interaction as purely antagonistic, additive or synergistic. There was a great amount of variation in terms of blebs and bridge blebs between treatments which means it is difficult to derive any association with the stressors, although there does seem to be similar modulation in response between 10 µM and 100 µM DON and blebbing/bridges are considered part of late stage apoptosis (Table 6.3). When considering the scored micronuclei and nucleoplasmic bridges it is apparent that there is an increase in aberrations due to dose of DON compared with the control, although RET yielded modulation of this effect. In isolation, RET caused an increase in aberrations until 100 µM when the amount decreased, due presumably to excessive crosslinking preventing mitosis. APH (0.5 µM) increased the incidence of nucleoplasmic aberrations at all concentrations of RET when in combination, except at 10 µM RET when the damage had seemingly reached a thresholded limit. A combination of DON doses with 0.1 µM MMC yielded a significant proportional increase in aberrations with the same dose-relationship as DON in isolation (F= 6.39, P<0.001, 74.2% of variation explained by DON dose, Figure 6.9).
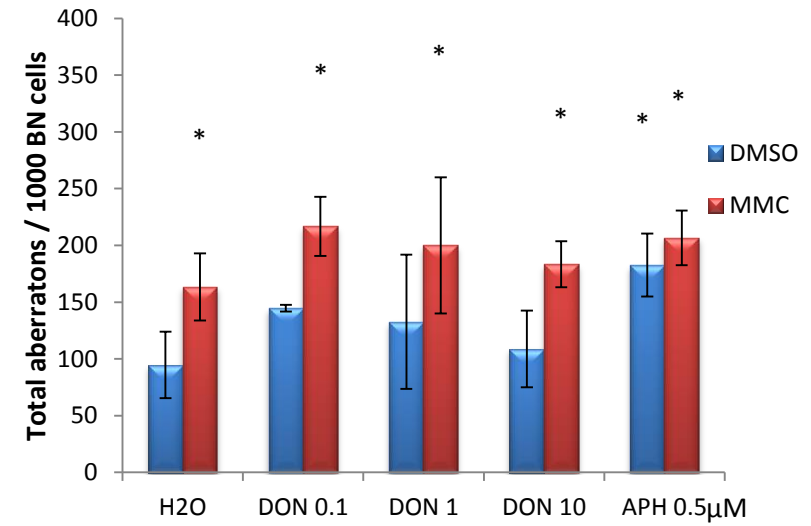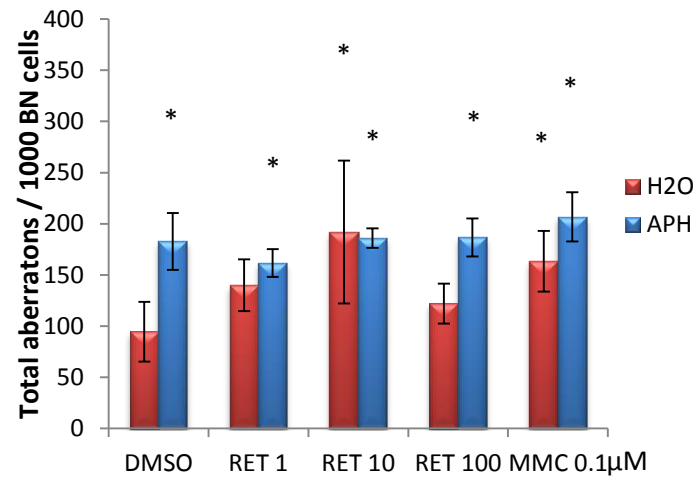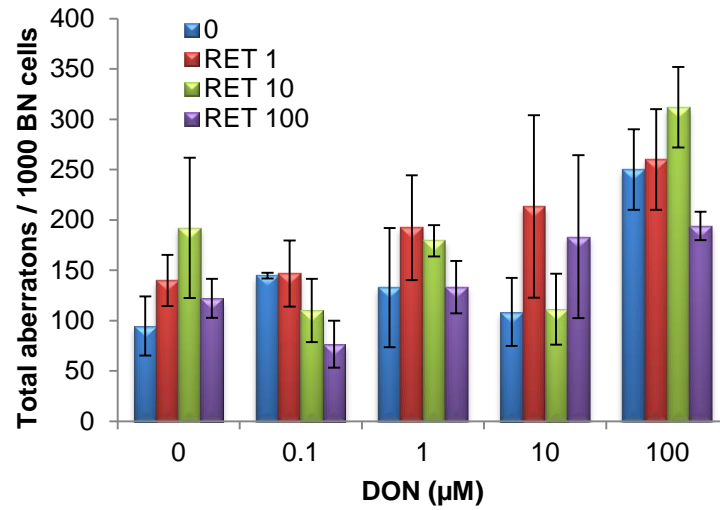
Figure 6. 9. The incidence of total nucleoplasmic aberrations following 48 h exposure of HepG2 cells to different concentrations of DON and RET (above left), RET and MMC with APH (above) or DON/APH and MMC (left) in isolation and in combination. Recorded aberrations include: micronuclei, nucleoplasmic bridges, blebs, bridge-blebs and mitotic catastrophe. The mean count per 1000 cytokinesis-blocked binucleated cells is shown with the standard error of triplicate experiments. Treatments yielding a significant increase in incidence over the control are shown with an asterisk (*).

6.3.5.5 Nuclear division index (NDI) assay
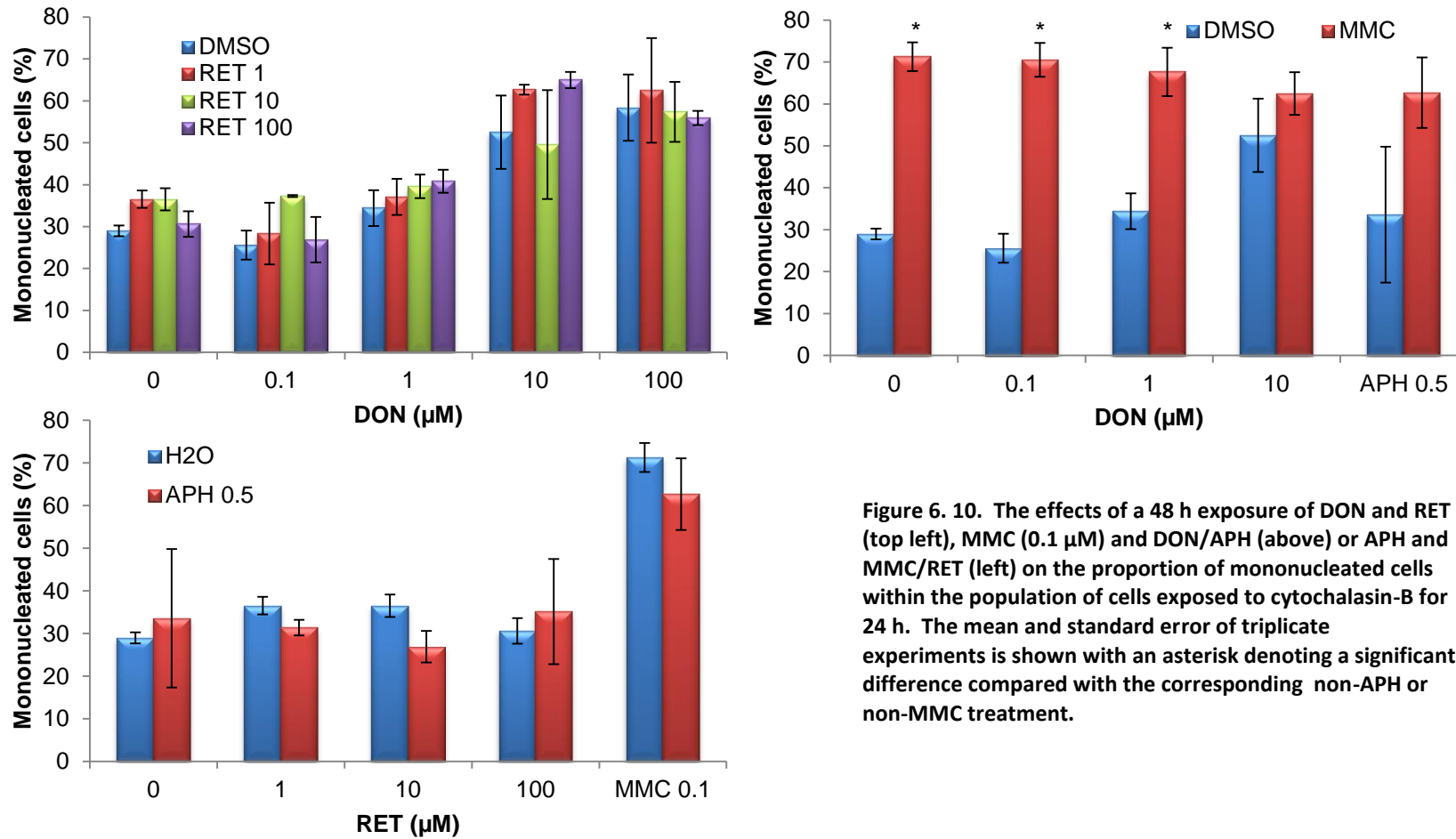
Figure 6. 10. The effects of a 48 h exposure of DON and RET (top left), MMC (0.1 µM) and DON/APH (above) or APH and MMC/RET (left) on the proportion of mononucleated cells within the population of cells exposed to cytochalasin-B for 24 h. The mean and standard error of triplicate experiments is shown with an asterisk denoting a significant difference compared with the corresponding non-APH or non-MMC treatment.

The proportion of cells undertaking mitosis can be determined by counting the number of

mono-, bi-, tri- and tetranucleated cells following the blocking of cytokinesis with

cytochalasin-B. A higher proportion of mononucleated cells equates to a reduction in

cellular turnover. An increase in DON concentration reduces cellular turnover

significantly (F = 22.19, $P<0.001$) across all treatments with 64.4% of variance associated

with DON dose. RET (F = 0.80, P = 0.50) either in isolation or in combination with DON (F =

0.85, $P<0.60$) did not surprisingly restrict mitotic turnover. MMC (0.1 µM) reduced cell

turnover by a factor of 2 for low doses of RET, yet high doses of DON and APH restricted

this effect (Figure 6.10).

6.4.3 MTT assay
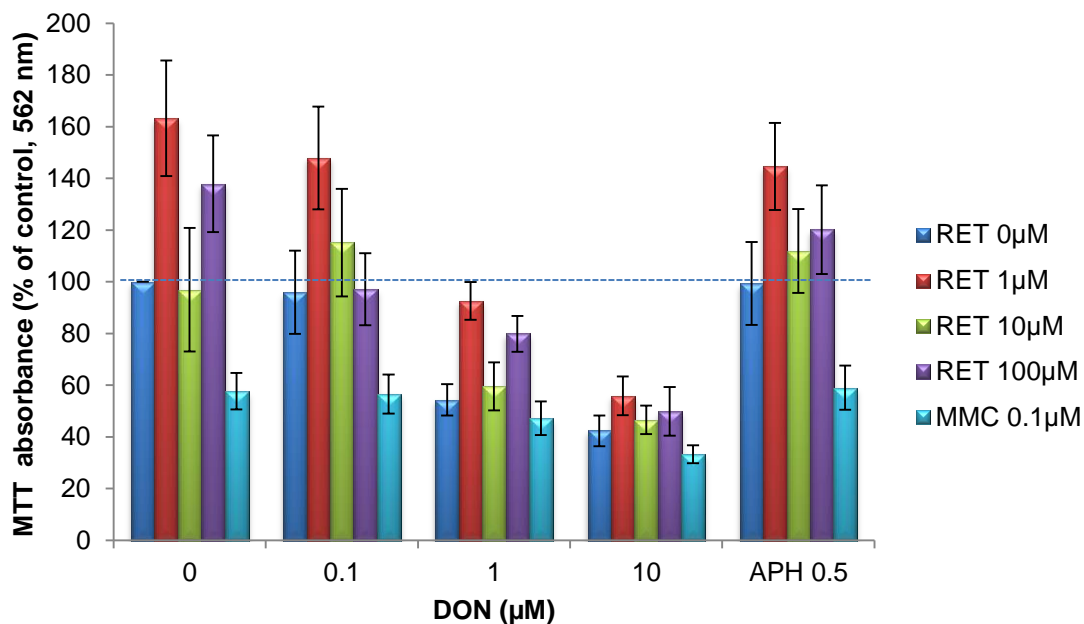
Figure 6.11. MTT absorbance as indicator of cellular vitality following a 48 h exposure to toxin. Means of triplicate wells and triplicate experiments shown with SE.

Cellular viability (including mitochondrial vitality) and cellular proliferation measured by

MTT metabolism was significantly reduced with concentration of DON (F = 15.25, $p<0.001$)

with an indication of antagonistic effects found with RET at low doses (Figure 6.11). No

synergism was determined using a GLM (R-Sq = 60.6%). MMC at 0.1 µM showed a 50%

reduction or more over the control and 0.5 µM APH gave a similar response to low doses

of DON. The MTT assay was also used for DON-3-glucoside and patulin for comparison in

a separate validation experiment (Figure 6.12). DON (Pearson correlation -0.720, 0.008)

was comparable in terms of toxicity to Patulin (-0.918, <0.001), an RNA synthesis inhibitor

produced by *Penicillium expansum*. The D3G dose tested did not elicit a reduction in MTT

metabolism in HepG2 cells compared with DON, indicating that the glucoside conjugate is

not only instrumental in preventing toxicosis in plants, but remains un-cleaved in HepG2

cells and is therefore innocuous over 48 h.
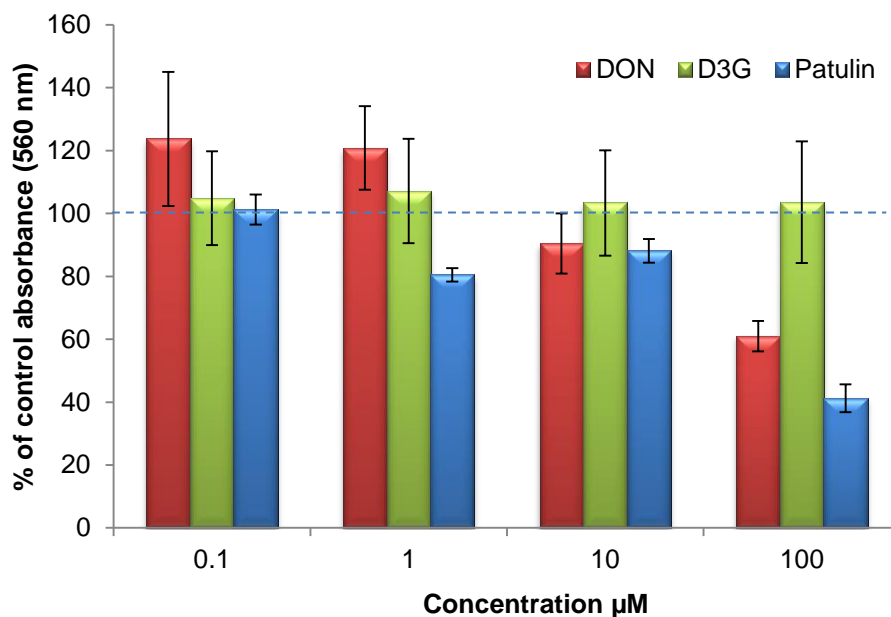
Figure 6.12. MTT absorbance as indicator of cellular vitality following a 48 h exposure to DON, D3G and Patulin (*Penicillium expansum*) for assay validation. Means of triplicate wells and triplicate experiments shown with SE.

6.4.4 Lactate dehydrogenase leakage assay
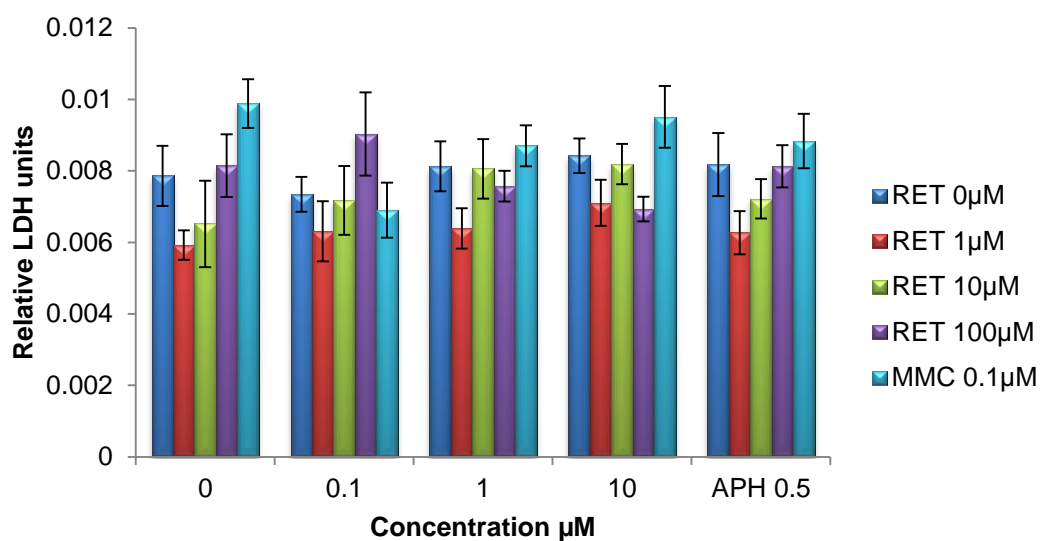
Figure 6.13. Lactate dehydrogenase measurements in the medium of HepG2 cells exposed to different combinational concentrations of DON and RET for 48 h. Relative LDH units is related to a standard curve constructed using a purchased standard solution. The mean of three experiments is shown with the SE.
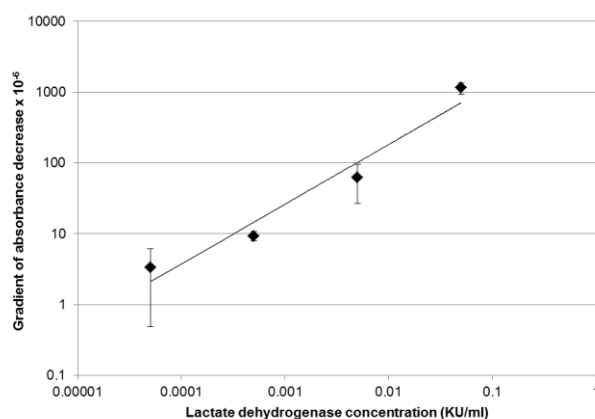
Figure 6.14. Standard curve of a lactate dehydrogenase concentration against the gradient decrease of NADH absorbance (362 nm, *i. e.* the rate of NADH to NAD$^+$ conversion). The equation $8829.9x^{0.8427}$ fits 94.5% of the data.

Lactate dehydrogenase measured via NADH to NAD$^+$ conversion did not show any significant response with the treatments used in the current study (ANOVA; F = -0.26, *P* = 0.802). This agrees well with the trypan blue membrane integrity result which suggests cellular leakage was not an endpoint associated with the DON/RET doses used.

6.4.5 Carboxyfluorescein diacetate assay (flow cytometry)
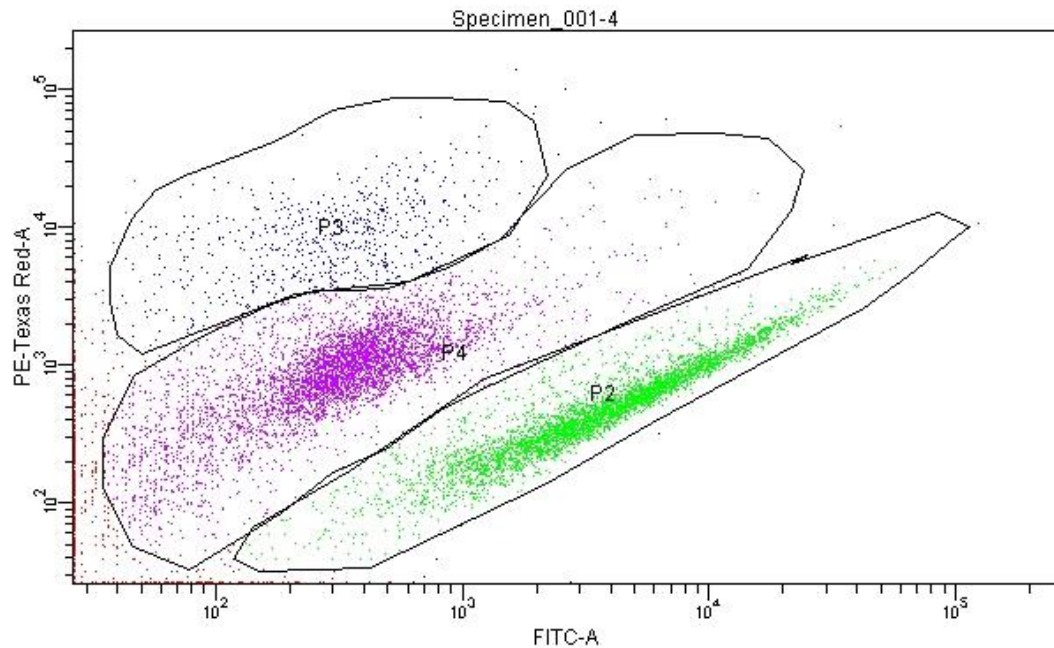
Figure 6.15. Example of selection gates used for CFDA flow-cytometry experiment. P2 equates to metabolically competent cells, P3 to advanced membrane damage and P4 to compromised membranes. Cells were exposed to fluorescein diacetate and propidium iodide following a 48 h 0.1 µM MMC treatment exposure.

The proportion of cells unable to metabolise CFDA as measured with flow cytometry followed a dome like response with concentrations of DON with 1 µM yielding an increase in FITC positives and only 100 µM yielding a decrease compared with the control (1-way ANOVA, F = 17.78, P<0.001, Dunnett's pairwise comparison with control at P<0.05). An increase in RET dose actually increased the proportion of FITC positive cells with a combinational exposure having antagonistic effects at low DON levels and a diminished influence at 100 µM DON. DON seemingly played a similar role with MMC as 1 µM-10 µM increased FITC positives, only for 100 µM to reduce it again, but not down to the MMC 0.1 µM or 10 µM levels. RET decreased the proportion of FITC positive cells when treated with 0.5 µM and 1.5 µM APH in a dose dependant dome-like trend.
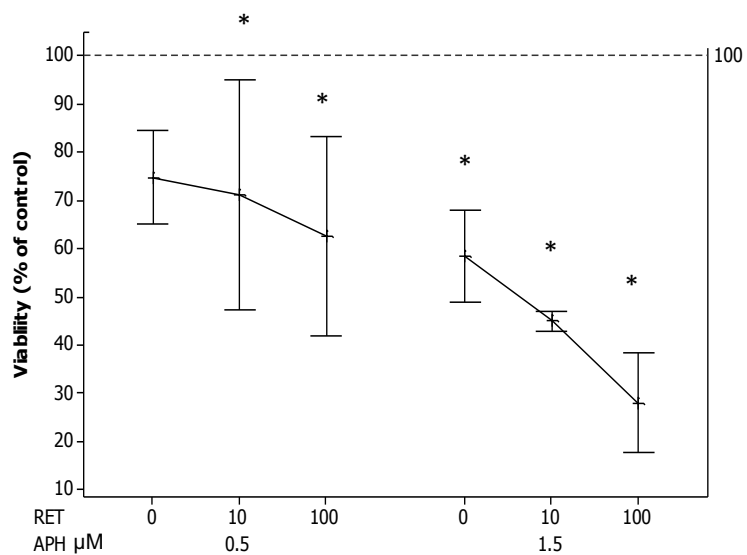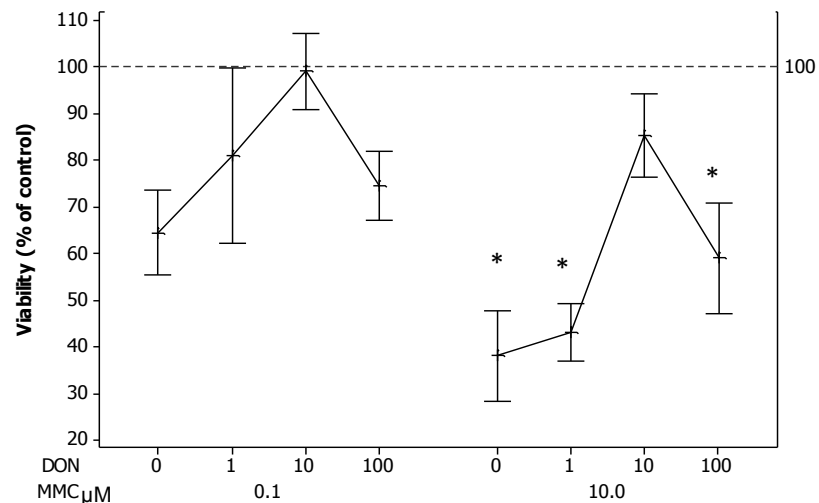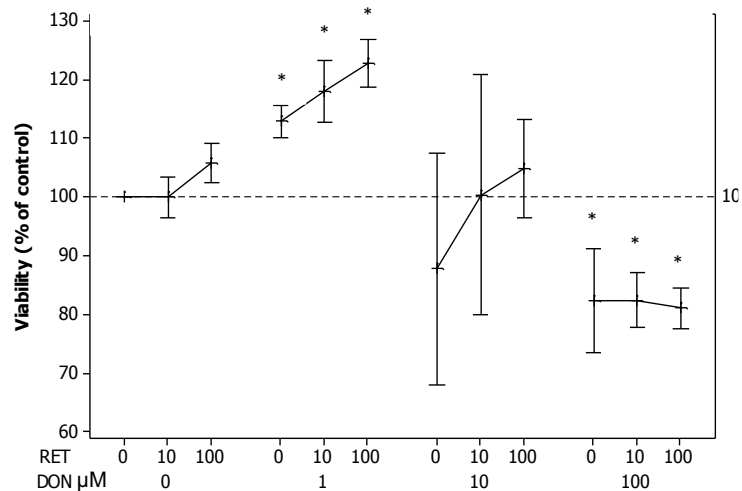
Figure 6.16. CFDA assay results determined using flow cytometry following a 48 h exposure HepG2 cells to DON and RET (µM) (top left), DON and MMC (above) or RET and APH (left). 10000 events were gated using FITC for healthy cells and propidium iodide as indicative of loss of membrane integrity with the mean category percentage shown of 3 experiments with the standard error and an asterisk denoting a significant difference to the control at *P*<0.05.

6.4.6 Carboxyfluorescein diacetate assay (fluorescent microscopy)
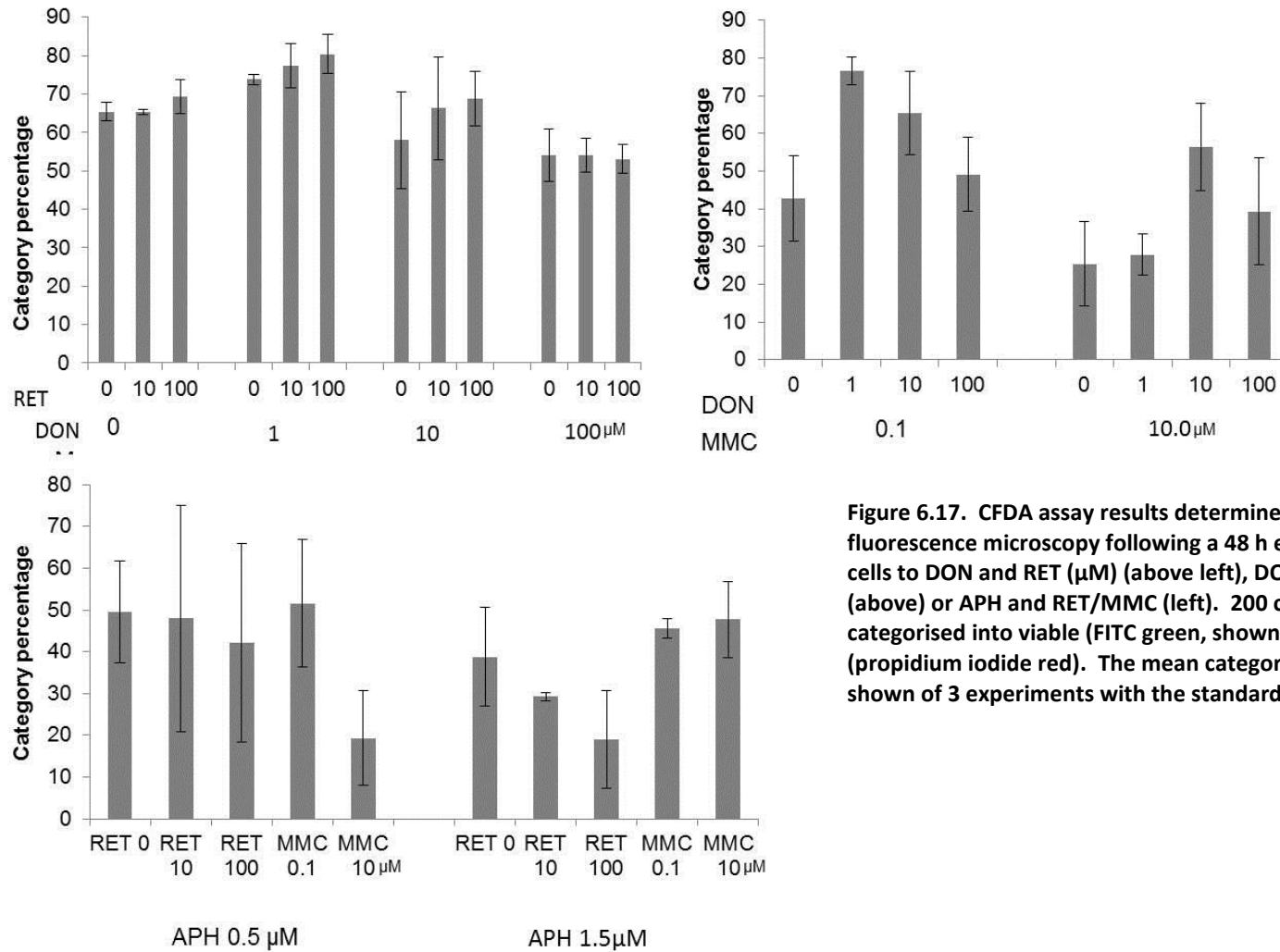
Figure 6.17. CFDA assay results determined using fluorescence microscopy following a 48 h exposure HepG2 cells to DON and RET (μM) (above left), DON and MMC (above) or APH and RET/MMC (left). 200 cells were categorised into viable (FITC green, shown) and non-viable (propidium iodide red). The mean category percentage shown of 3 experiments with the standard error.

When the same population of cells that were analysed by flow cytometry were observed

and categorised by light microscopy a similar dose dependant relationship was found with

RET and DON and the other treatments.

6.4.7 Annexin V-FITC apoptosis assay (flow cytometry)
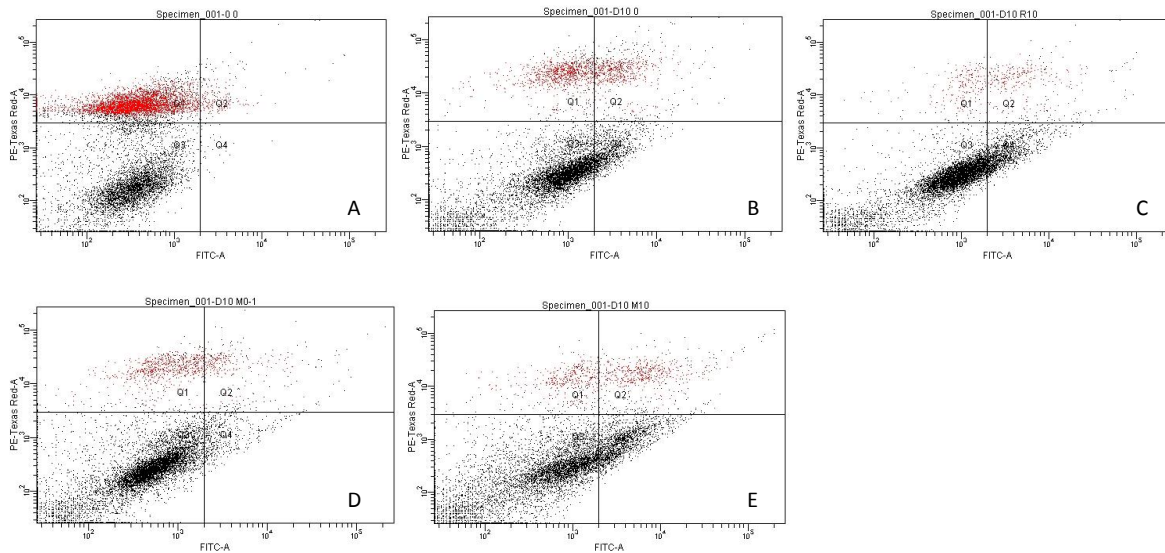
Figure 6.18. Flow cytometry plots of Annexin V-FITC (black) and propidium iodide (red) stained HepG2 cells following 48 h exposure to: A, control; B, DON 10 μM; C, DON 10 μM + RET 10 μM; D, DON 10 μM + MMC 0.1 μM; E, DON 10 μM + MMC 10 μM.
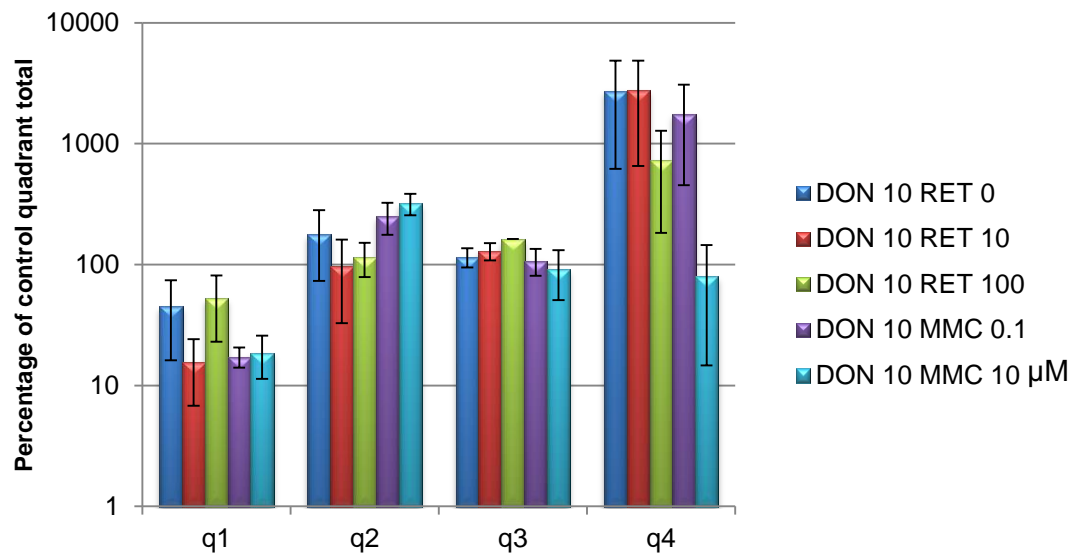
Figure 6.19. Flow cytometry data grouped by quadrants following treatment of HepG2 cells to DON, RET and MMC. Apoptotic cells (V-FITC) fluoresce green with an increase in intensity resulting in an increase in cells in Q4. An increase in Q2 indicates an increase in apoptosis and compromised membrane integrity. 10,000 cells were analysed for each triplicate experimental treatment with the mean percentage of control treatment quadrant value shown with the standard error.

The flow cytometry results derived from annexin V5 treated cells indicate all treatments apart from MMC 10 µM tend to increase the proportion of cells in quadrant 4, an arbitrary method of dividing scatter data in this case indicating high FITC intensity cells representing cells presenting annexin V5 in the process of apoptosis. MMC 10 µM treatment reduces this intensity and shifts the cluster to an increase in red fluorescence.

6.4.8 Annexin V-FITC apoptosis assay (fluorescence microscopy)
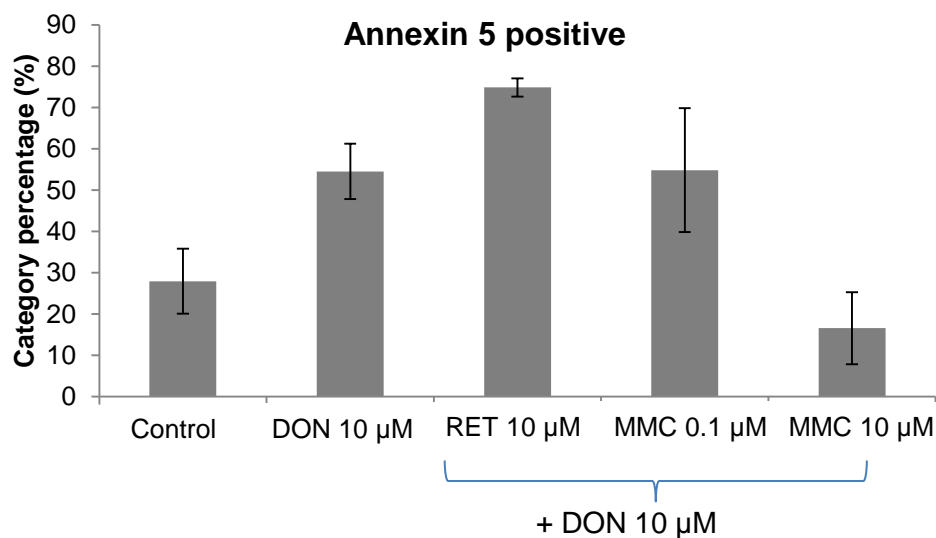
Figure 6.20. Percentage of apoptotic cells exhibiting Annexin V5 as determined using fluorescent microscopy. Cells analysed were exposed for 48 h to combinations of DON (10 µM), RET and MMC. The mean is shown with the standard error of triplicate experiments (200 cells).

The proportion of those cells displaying phosphatidylserine on the cytoplasmic surface of cells suggests the initiation of apoptosis was around 25% in the untreated cells. The addition of 10 µM DON increases this to over 50% and the addition of 10 µM RET increases this to over 75%. Replacing RET with MMC 0.1 µM yielded similar response to DON in isolation, although a necrotic dose of 10 µM reduced apoptosis induction presumably through the damage exceeding checkpoint capacity and damaging the membranes.

6.4.9 Trypan blue and monolayer cell density

Cellular membrane integrity as determined by trypan blue exclusion indicates that only

the highest doses of DON (GLM 67.8%, F = 19.6, $P<0.001$) (Figure 6.21). The evidence

suggests damage inflicted is not membrane associated, but subtly affects other cellular

aspects, for example in the nucleus.
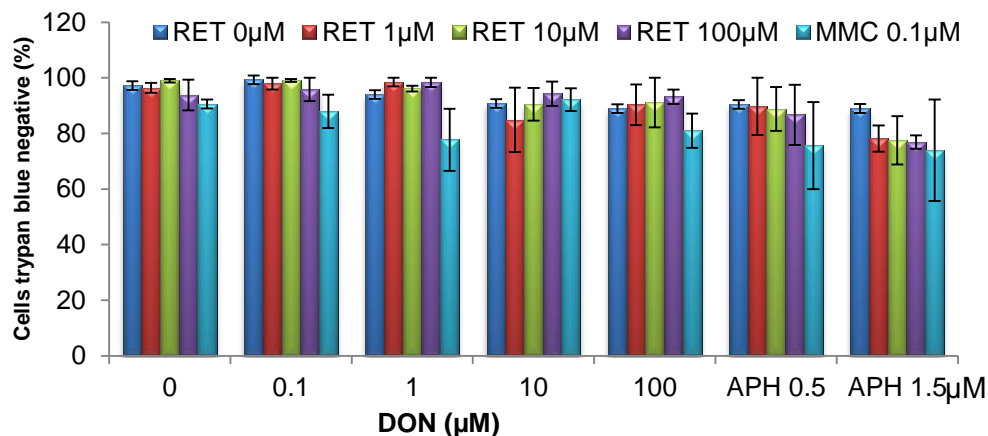
Figure 6.21. Percentage of cells excluding trypan blue stain following a 48 h toxin exposure. Means of triplicate wells and triplicate experiments shown with SE.
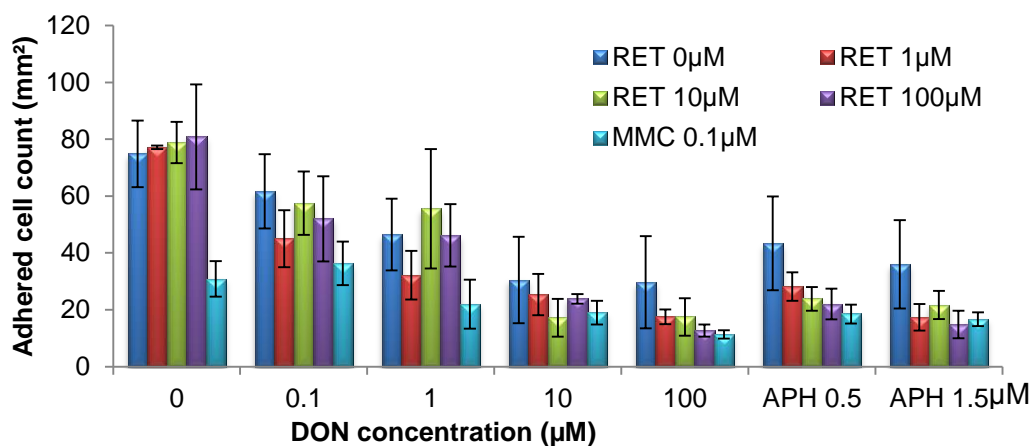
Figure 6.22. The cell count per mm^2 of HepG2 cells seeded at 5 x 10^4 cells/ml and incubated for 24 h followed by 48 h exposure to toxins. Means of triplicate wells and; triplicate experiments shown with SE.

The results of the cell count experiment broadly agree with that of the protein assay with

DON concentration the only significant stressor affecting cell proliferation (GLM, R-Sq =

65.2%, F = 17.38, $p<0.001$). The APH treatment reduced cell number as did the MMC

dose which indicates cell number and amount of protein may not be the definitive story and cell morphology and size may provide more information than these two endpoints.

6.4.10 Protein assay
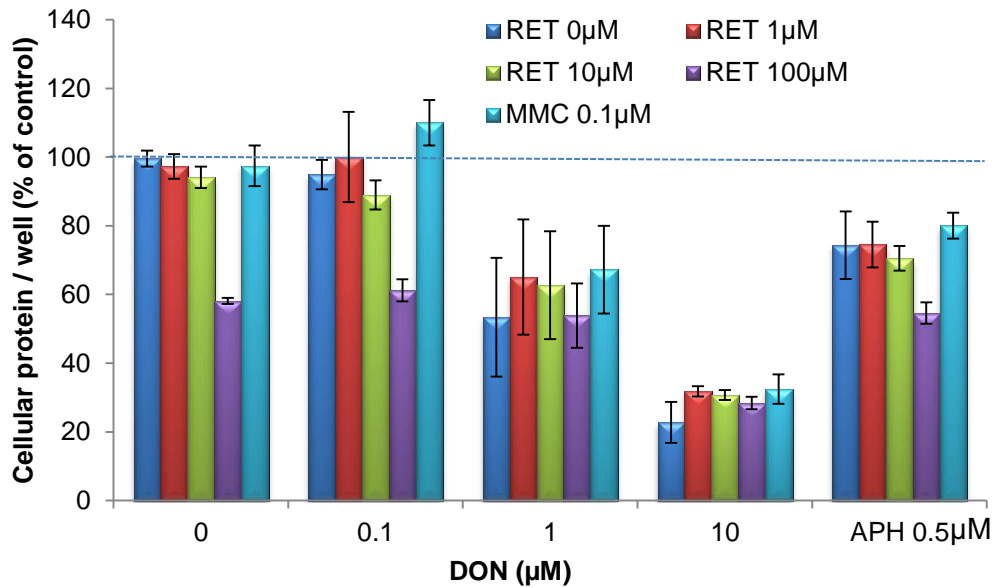
Figure 6.23. Cellular protein contents per well following a 48 h toxin exposure. Means of triplicate wells and triplicate experiments shown with SE.

The effects of both toxins on cellular turnover measured by determining the total adhered cellular protein content of each well following 48 h of exposure indicates DON was the main contributor to reduced cellular turnover and protein content in each well (GLM, R-Sq = 83.8%, F = 47.14, $p<0.001$).   The protein assay results closely resemble that found with MTT metabolism confirming that both give a good measure of cellular vitality reinforced by absolute cell counts and NDI.

6.4.11 Proteomics

Analysing all the proteins by cluster analysis indicated that In general the RET/DON treatment yielded substantially different values across the whole proteome compared with the similarity found with the other treatments (Figure 6.24).
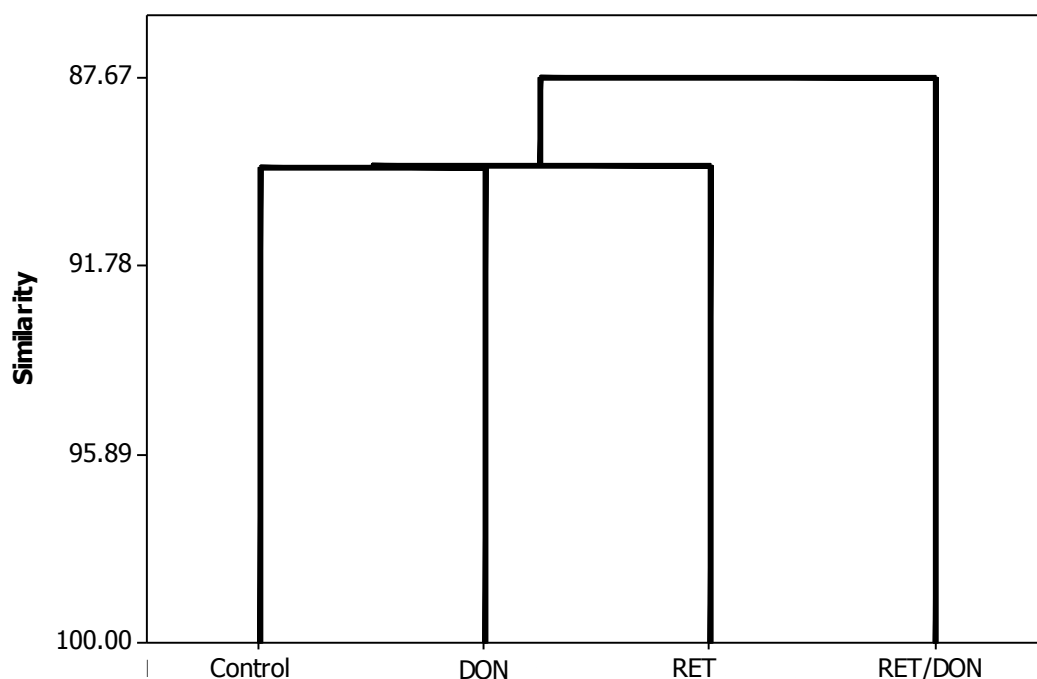
Figure 6.24. Dendrogram of 1749 protein groups determined with mass spectrometry following treatment of HepG2 cells for 48 h to DON (10 µM), RET (10 µM) or both (10 + 10 µM, pooled flasks in triplicate). The label-free peak intensity values for each protein group can be grouped and compared between treatments in terms of similarity by looking at the position of the joining partition.

It must be considered that prior to sample preparation the extracted protein was normalised between treatments to 1 mg/ml BSA levels and the pooled label-free quantification intensity recorded was very similar for each treatment (2.26, 2.17, 1.94 and 2.09 x 10^9 for control, DON (10 µM), RET (10 µM) and RET/DON (10 + 10 µM) respectively). It is only when we delve into specific protein groups that interesting differences can be elucidated.

Table 6.4. Summary of the key biological functions focussed on with proteomic analysis.

Biological function	Gene ontology term	Definition (UNIPROT)
Translational elongation	GO:0006414	The successive addition of amino acid residues to a nascent polypeptide chain during protein biosynthesis.
Protein biosynthesis	GO:0006412	The cellular metabolic process in which a protein is formed, using the sequence of a mature mRNA molecule to specify the sequence of amino acids in a polypeptide chain. Translation is mediated by the ribosome, and begins with the formation of a ternary complex between aminoacylated initiator methionine tRNA, GTP, and initiation factor 2, which subsequently associates with the small subunit of the ribosome and an mRNA. Translation ends with the release of a polypeptide chain from the ribosome.
Apoptosis	GO:0006915	Protein involved in apoptotic programmed cell death. Apoptosis is characterized by cell morphological changes, including blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation, and eventually death. Unlike necrosis, apoptosis produces cell fragments, called apoptotic bodies, which phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out onto surrounding cells and cause damage. In general, apoptosis confers advantages during an organism's life cycle.
Response to organic substance	GO:0010033	Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of an organic substance stimulus.
Membrane	GO:0016020	Protein which is membrane-bound or membrane-associated. A membrane is the layer which forms the boundary of cells and intracellular organelles. It is composed of two oriented lipid layers in which proteins are embedded and acts as a selective permeability barrier.
Cell cycle regulation	GO:0051726	Any process that modulates the rate or extent of progression through the cell cycle.

In an attempt to establish bulk proteome changes resulting from DON and RET treatment the identified protein groups were grouped by biological function, *i.e.* translational elongation (Table 6.4, 6.5), and the expression of the top 100 protein groups in size order related to the control were compared using cumulative ion count curves in addition to the construction of dendrograms to illustrate treatments which have significant protein expression differences (Figure 6.25). In addition, the 4 fold protein expression differences of particularly relevant functions are listed in Table 6.6 (with a full list in appendix 10.1/10.2).

When the proteins were grouped by using DAVID® it was readily apparent that the 5 most profuse proteins in each functional group were broadly similar between all treatments. Translational elongation, the aspect of protein synthesis known to be most impacted by DON exposure was found to be significantly enriched with DON and DON/RET, whilst both these groups had reduced quantity of proteins related to protein synthesis.

Apoptosis linked proteins were most numerous for DON, less so for DON/RET followed by RET. The control had the least quantity of apoptosis associated proteins as a whole. This is the same relationship concerning cell cycle regulation proteins, which are likely to be closely related to apoptosis induction. Membrane-bound /-associated protein provision was interesting as the top 5 proteins for the toxin treatments were skewed to mitochondrial associated enzymes for all treatments with broadly similar proportions of each, which could be considered housekeeping proteins within each proteome against which other fluctuations are compared. Bulk shifts in enrichment suggest RET to have a more equal spread of membrane proteins in terms of quantity compared with the control which had a larger proportion of higher count proteins, or the DON and DON/RET treatments which were consistently lower across the top 100 proteins identified. RET can be seen to reduce the protein expression related to a response to an organic substance, especially when combined with DON. This would have effects on general liver cell function including microsomal activity. The suite of proteins found at the highest levels in this gene ontology group for all treatments includes heat-shock proteins (similar presence), in addition to epoxide hydrolase (2-fold increase with DON, DON/RET) and NM23A, an important nucleoside synthesis and DNA endonuclease (3 to 4-fold less with RET/DON). The main observation is that these toxin treatments reduce enrichment of

proteins applicable to the response from organic substances, rather than eliciting an expected increase.

The overriding result regarding 4 fold deviations from control conditions (only universally expressed protein groups across treatments analysed) would be the sheer number of proteins increased by the DON treatment and amount reduced following DON and DON/RET exposure (Table 6.6, 6.7). DON exposure led to the 4-fold increase in 118 protein groups, whereas RET and RET/DON led to 20 and 18 respectively. 4-fold reductions in 51, 9 and 73 protein groups were found with DON, RET and RET/DON respectively. A few of the most expressed and relevant identities are evaluated further. Actin (gamma 1), an omnipresent cytoskeleton protein which has been shown by Coulombe Jr *et al.* (1999) to be the most likely protein involved in DNA-protein crosslinks by PAs and MMC, was found extensively in all treatments without significant variation. Microsomal and cytosolic glutathione-s-transferases (GST) are membrane bound phase II excretion enzymes crucial to xenobiotic detoxification within the cell (Strange and Hayes, 2000). Regarding the GST family, only GST-1 was found to be present in elevated amounts (9 fold in DON) compared with GST-2 and 3 which were equivalent to the control. Glutathione reductase, an enzyme involved in ROS reduction within the cell on the other hand was only increased in the RET/DON combination. Interestingly, DON exposure led to an increase in mitochondrial membrane proteins, specifically those involved in respiration (cytochrome-c oxidase *etc.*). Whilst cellular turnover may be reduced, it seems the metabolic status of the cell is increased by DON leading to potential ROS production and apoptosis induction.

In terms of cytotoxicity, DON and RET/DON produced the greatest expression of proteins associated with apoptosis, an effect seen with the other experimental endpoints. The lack of enrichment of mitogen-activated protein kinase 1 (MAPK-1) suggests this specific pathway of ROS-induced apoptosis is not employed by the cells. A disruption in the balance of apoptotic to anti-apoptotic protein groups will have a pronounced effect on cell functionality. UDP-glucuronosyltransferase saw a three-fold increase for DON and RET and increased by half for RET/DON. Whilst this is less than a 4-fold difference, it does confirm presence of this phase II system in HepG2 and tentatively this could be viewed as an upregulated mechanism for both RET and DON excretion, which is noteworthy considering this is the main route of DON excretion in mammals (He *et al.*, 2010b, Maul *et al.*, 2014). The most enriched protein group in the DON treated cells with a 57x increase, was found to be catenin δ1; a cell-cell adhesion protein and DNA-transcription regulator. In RET the catenin response was 5x and in RET/DON it was at control levels. Lactate dehydrogenase (LDH) was found to be very low for all treatments (at least 500x less than control). Unfortunately this cannot be linked to LDH leakage or cell density data and remains unexplained for now.

Table 6.5. The top-5 expressed proteins in each Gene-ontology category for each treatment. Protein groups of especial relevance are highlighted with an asterisk.

Biological function	Control	
	Protein group	Ion intensity
Translational elongation (1)	protein disulfide isomerase family A, member 3	11310000
2	annexin A6	10692000
3	heterogeneous nuclear ribonucleoprotein A1-like 3	9839600
4	eukaryotic translation elongation factor 1 alpha-like 7	6949400
5	insulin-like growth factor 2 mRNA binding protein 1	4434500
Protein biosynthesis (1)	aminoacyl tRNA synthetase complex-interacting multifunctional protein 2	6949400
2	ribosomal protein S16 pseudogene 1	5245400
3	threonyl-tRNA synthetase	4342300
4	eukaryotic translation initiation factor 2B, subunit 4 delta, 67kDa	3385400
5	glutamyl-tRNA synthetase 2, mitochondrial (putative)	3291500
Membrane (1)	ATPase, H+ transporting, lysosomal accessory protein 1	78014000
2	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	51901000
3	phospholipase C, eta 1	31067000
4	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1	25321000
5	prolyl 4-hydroxylase, beta polypeptide	18303000
Apoptosis (1)	hypothetical gene supported by AF216292; NM_005347; heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	49712000
2	API5-like 1; apoptosis inhibitor 5	33711000
3	non-metastatic cells 1, protein (NM23A) expressed in; NME1-NME2 readthrough transcript	27976000
4	tubulin, beta 2C	26436000
5	heat shock 10kDa protein 1 (chaperonin 10)	20699000
Response to organic substance (1)	non-metastatic cells 1, protein (NM23A) expressed in; NME1-NME2 readthrough transcript	27976000
2	heat shock 10kDa protein 1 (chaperonin 10)	20699000
3	heat shock 70kDa protein 8	11843000
4	protein disulfide isomerase family A, member 3	11310000
5	annexin A6	10692000
Cell cycle regulation (1)	histone cluster 1, H4l; histone cluster 1, H4k	103900000
2	ATPase, H+ transporting, lysosomal accessory protein 1	78014000
3	actin, gamma 1	60755000
4	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	51901000
5	hypothetical gene supported by AF216292; NM_005347; heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	49712000
Total cell protein (1)	histone cluster 1, H2bb	183870000
2	general transcription factor II, i; general transcription factor II, i, pseudogene	156790000
3	histone cluster 1, H4l; histone cluster 1, H4k;	103900000
4	ATPase, H+ transporting, lysosomal accessory protein 1	78014000
5	histone cluster 2, H2aa3; histone cluster 2, H2aa4	64870000

Biological function	DON	
	Protein group	Ion intensity
Translational elongation (1)	heterogeneous nuclear ribonucleoprotein A1-like 3	25517000
2	eukaryotic translation elongation factor 1 alpha-like 7	11677000
3	ribosomal protein S3A pseudogene 5	5553300
4	ribosomal protein S3 pseudogene 3; ribosomal protein S3	5475800
5	annexin A6	5164700
Protein biosynthesis (1)	aminoacyl tRNA synthetase complex-interacting multifunctional protein 2; stromal antigen 3	11677000
2	ribosomal protein S19 pseudogene 3; ribosomal protein S19	5553300
3	ribosomal protein L18	5475800
4	ribosomal protein S4, Y-linked 1	4390400
5	ribosomal protein S5	4376400
Membrane (1)	ATPase, H+ transporting, lysosomal accessory protein 1	56494000
2	solute carrier family 25 (mitochondrial carrier; adenine nucleotide)	26273000
3	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1,	25760000
4	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	23802000
5	voltage-dependent anion channel 1	19722000
Apoptosis (1)	API5-like 1; apoptosis inhibitor 5	41848000
2	non-metastatic cells 1, protein (NM23A) expressed in; NME1-NME2 readthrough transcript	26751000
3	tubulin, beta 2C	20746000
4	hypothetical gene supported by AF216292; NM_005347; heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	16673000
5	Prohibitin	13111000
Response to organic substance (1)	non-metastatic cells 1, protein (NM23A) expressed in; NME1-NME2 readthrough transcript	26751000
2	heat shock 70kDa protein 8	13941000
3	heat shock protein 90kDa alpha (cytosolic), class B member 1	10531000
4	heat shock 10kDa protein 1 (chaperonin 10)	7063000
5	epoxide hydrolase 1, microsomal (xenobiotic)*	6973300
Cell cycle regulation (1)	histone cluster 1, H4l	94259000
2	ATPase, H+ transporting, lysosomal accessory protein 1	56494000
3	actin, gamma 1	29462000
4	non-metastatic cells 1, protein (NM23A) expressed in; NME1-NME2 readthrough transcript	26751000
5	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	25760000
Total cell protein (1)	general transcription factor II, i; general transcription factor II, i, pseudogene	111920000
2	histone cluster 1, H4	94259000
3	histone cluster 1, H2bb	76123000
4	histone cluster 2, H2aa3; histone cluster 2, H2aa4	61655000
5	ATPase, H+ transporting, lysosomal accessory protein 1	56494000

Biological function	RET	
	Protein group	Ion intensity
Translational elongation (1)	protein disulfide isomerase family A, member 3	8578300
2	insulin-like growth factor 2 mRNA binding protein 1	5945000
3	eukaryotic translation elongation factor 1 alpha-like 7	5658600
4	heterogeneous nuclear ribonucleoprotein A1-like 3	5626900
5	Tu translation elongation factor, mitochondrial	4572100
Protein biosynthesis (1)	ribosomal protein S16 pseudogene 1	10550000
2	aminoacyl tRNA synthetase complex-interacting multifunctional protein 2	5658600
3	threonyl-tRNA synthetase	4572100
4	eukaryotic translation initiation factor 2B, subunit 4 delta, 67kDa	4075000
5	ribosomal protein S27 pseudogene 29	4064400
Membrane (1)	ATPase, H+ transporting, lysosomal accessory protein 1	71751000
2	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	38328000
3	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1,	24337000
4	solute carrier family 25 (mitochondrial carrier; adenine nucleotide)	14292000
5	voltage-dependent anion channel 1; similar to voltage-dependent anion	12527000
Apoptosis (1)	hypothetical gene supported by AF216292; NM_005347; heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	55566000
2	heat shock protein 90kDa beta (Grp94), member 1	25106000
3	API5-like 1; apoptosis inhibitor 5	25078000
4	non-metastatic cells 1, protein (NM23A) expressed in; NME1-NME2 readthrough transcript	18668000
5	tubulin, beta 2C	17174000
Response to organic substance (1)	non-metastatic cells 1, protein (NM23A) expressed in; NME1-NME2 readthrough transcript;	18668000
2	heat shock 70kDa protein 8	14014000
3	heat shock 10kDa protein 1 (chaperonin 10)	13092000
4	heat shock protein 90kDa alpha (cytosolic), class B member 1	9208400
5	protein disulfide isomerase family A, member 3	8578300
Cell cycle regulation (1)	histone cluster 1, H4l	116790000
2	ATPase, H+ transporting, lysosomal accessory protein 1	71751000
3	hypothetical gene supported by AF216292; NM_005347; heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	55566000
4	actin, gamma 1	50923000
5	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	38328000
Total cell protein (1)	histone cluster 1, H4lb	116790000
2	histone cluster 1, H2bb	108540000
3	histone cluster 2, H2aa3; histone cluster 2, H2aa4	105900000
4	ATPase, H+ transporting, lysosomal accessory protein 1	71751000
5	H3 histone, family 3B (H3.3B)	57523000

Biological function	RET/DON	
	Protein group	Ion intensity
Translational elongation (1)	eukaryotic translation elongation factor 1 alpha-like 7	12091000
2	heterogeneous nuclear ribonucleoprotein A1-like 3	9169500
3	ribosomal protein L4; ribosomal protein L4 pseudogene 5; ribosomal protein L4 pseudogene 4	7278800
4	insulin-like growth factor 2 mRNA binding protein 1	6957200
5	annexin A6	6658400
Protein biosynthesis (1)	aminoacyl tRNA synthetase complex-interacting multifunctional protein 2; stromal antigen 3-like 3	12091000
2	glutamyl-tRNA synthetase 2, mitochondrial (putative)	5435100
3	glutaminyl-tRNA synthetase	5095200
4	eukaryotic translation initiation factor 2B, subunit 4 delta, 67kDa	4833800
5	ribosomal protein L11	4306000
Membrane (1)	ATPase, H+ transporting, lysosomal accessory protein 1	48220000
2	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	42414000
3	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1	29454000
4	ribophorin I	14825000
5	coiled-coil domain containing 90B	13084000
Apoptosis (1)	tubulin, beta 2C	27689000
2	API5-like 1; apoptosis inhibitor 5	20380000
3	hypothetical gene supported by AF216292; NM_005347; heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	19909000
4	Prohibitin	9215400
5	synovial apoptosis inhibitor 1, synoviolin	7146400
Response to organic substance (1)	heat shock 70kDa protein 8	19581000
2	heat shock protein 90kDa alpha (cytosolic), class B member 1	17986000
3	insulin-like growth factor 2 mRNA binding protein 1	6957200
4	annexin A6	6658400
5	epoxide hydrolase 1, microsomal (xenobiotic)*	6282600
Cell cycle regulation (1)	histone cluster 1, H4l	107880000
2	ATPase, H+ transporting, lysosomal accessory protein 1	48220000
3	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	42414000
4	actin, gamma 1	38779000
5	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	29454000
Total cell protein (1)	histone cluster 1, H2bb	183870000
2	histone cluster 1, H4l	103900000
3	H3 histone, family 3B (H3.3B)	57014000
4	histone cluster 2, H2aa3; histone cluster 2, H2aa4	64870000
5	ATPase, H+ transporting, lysosomal accessory protein 1	78014000
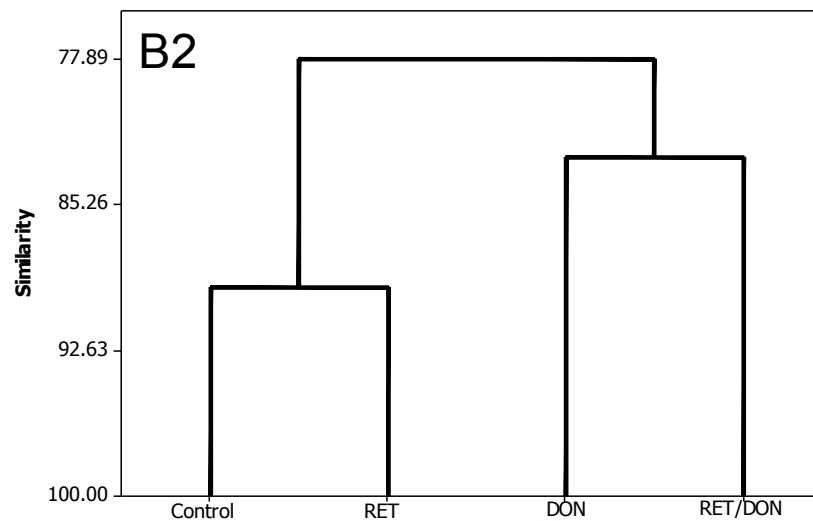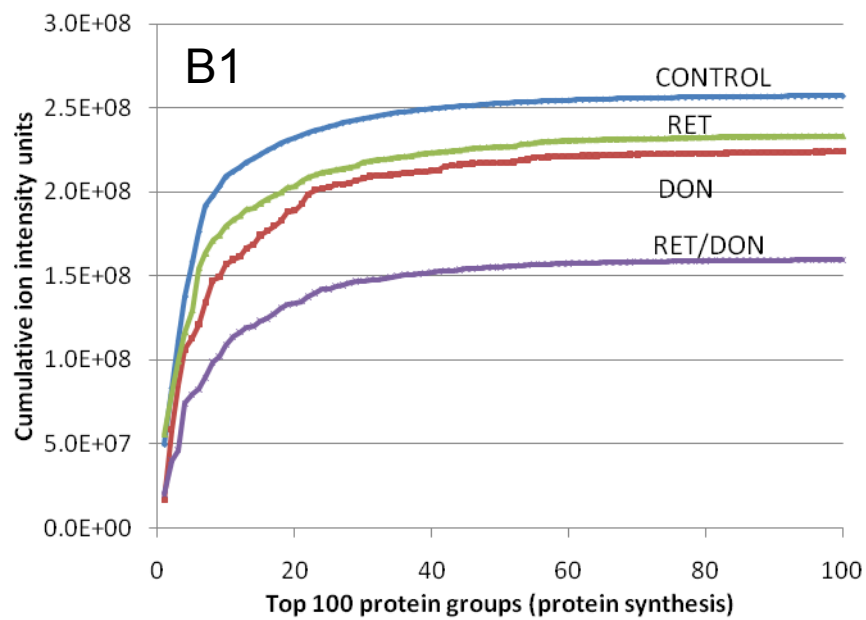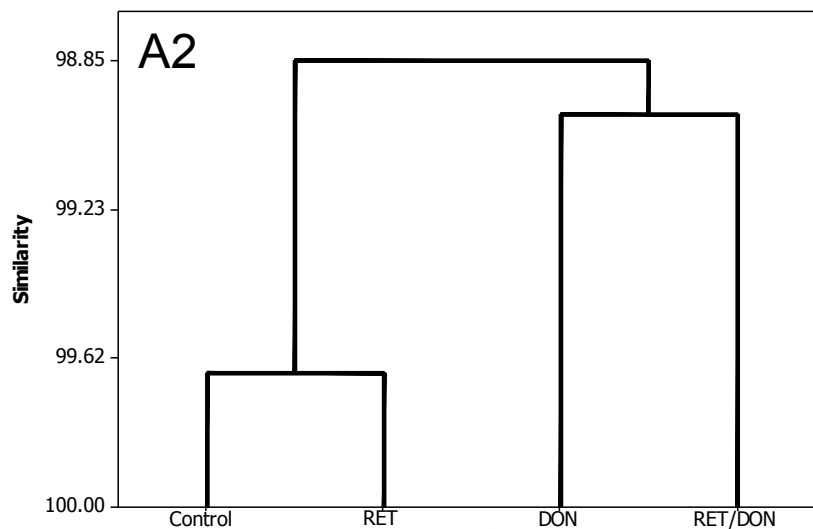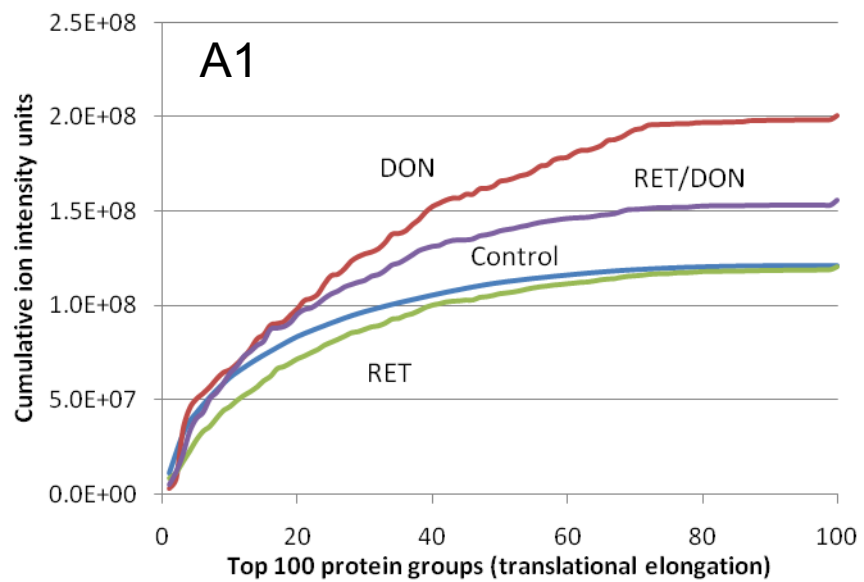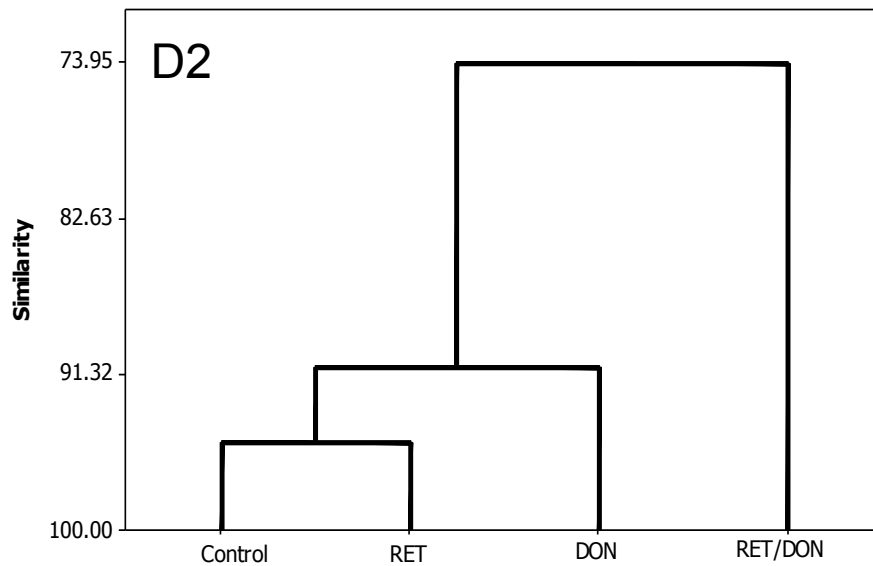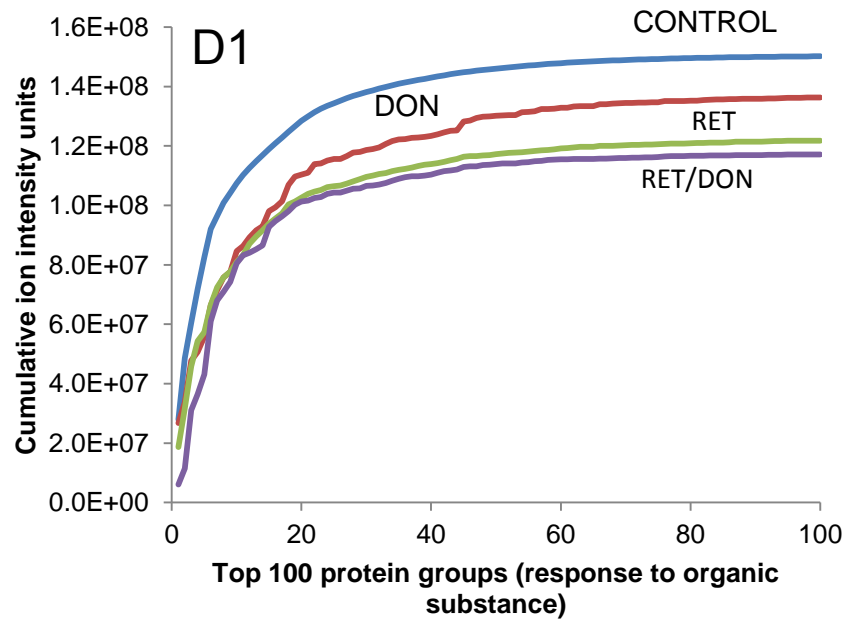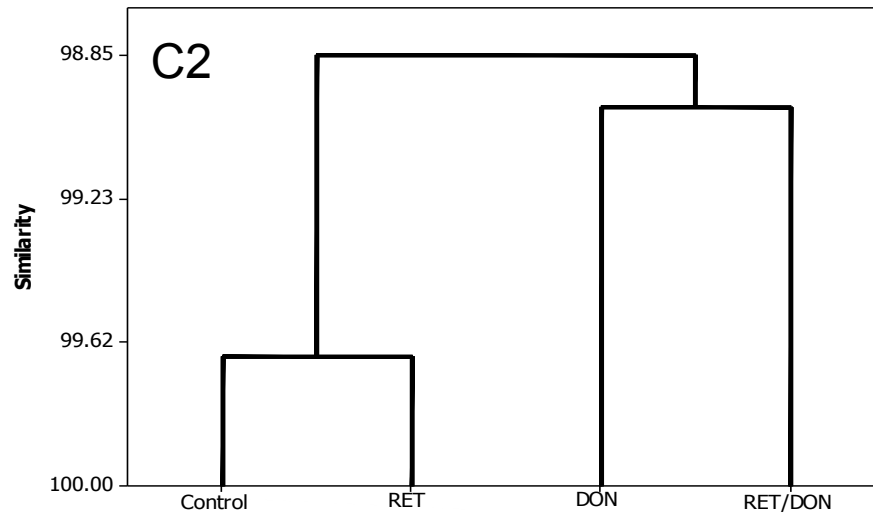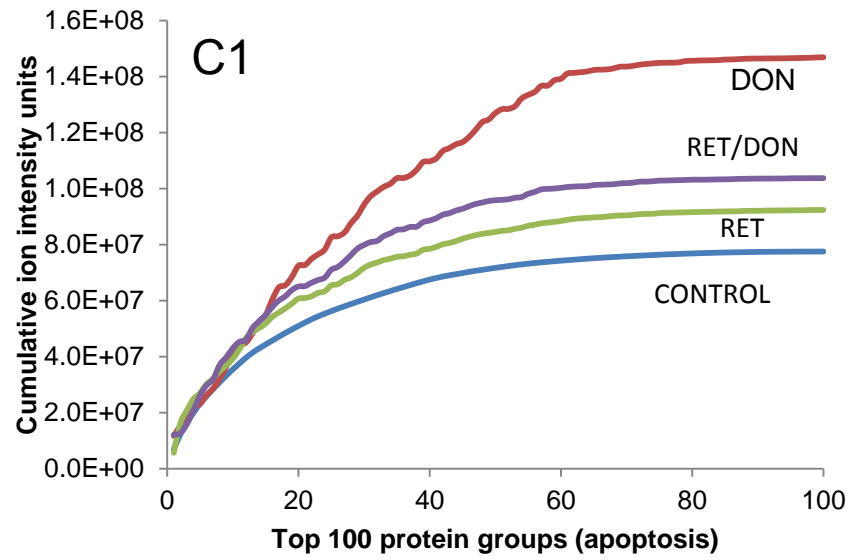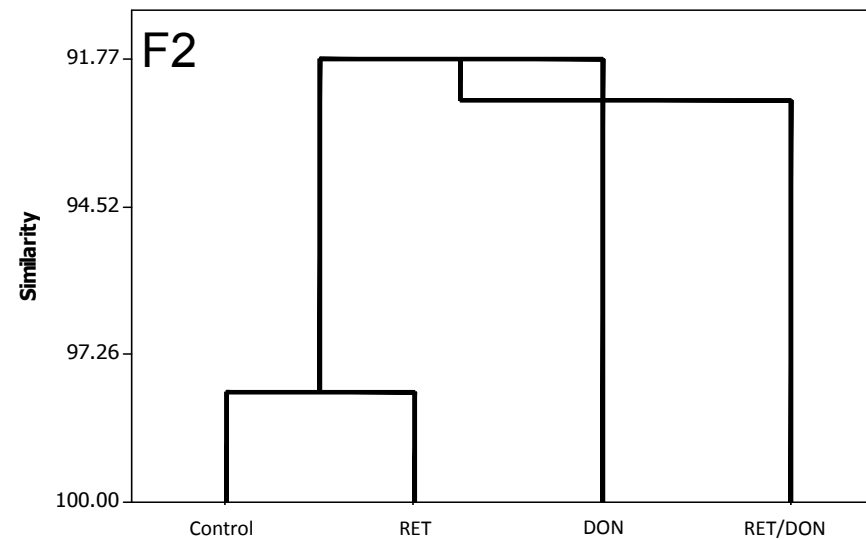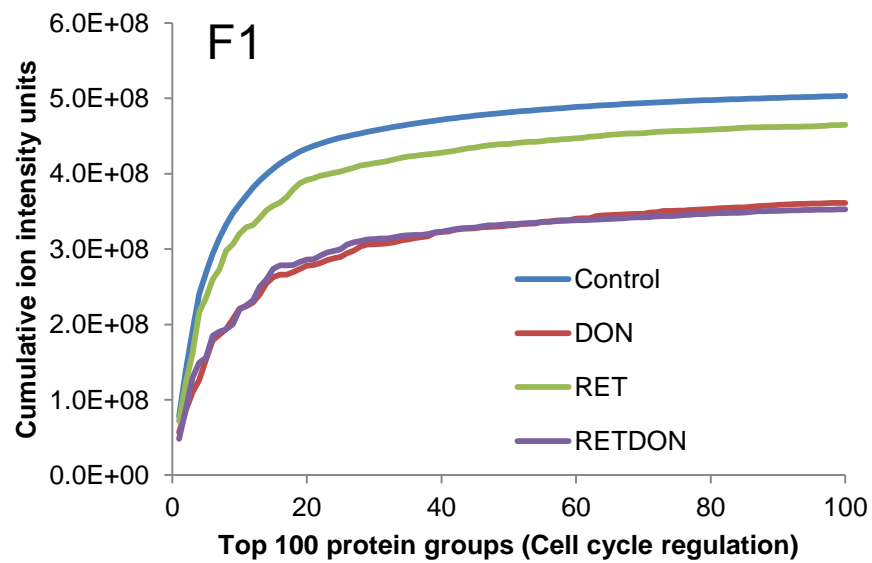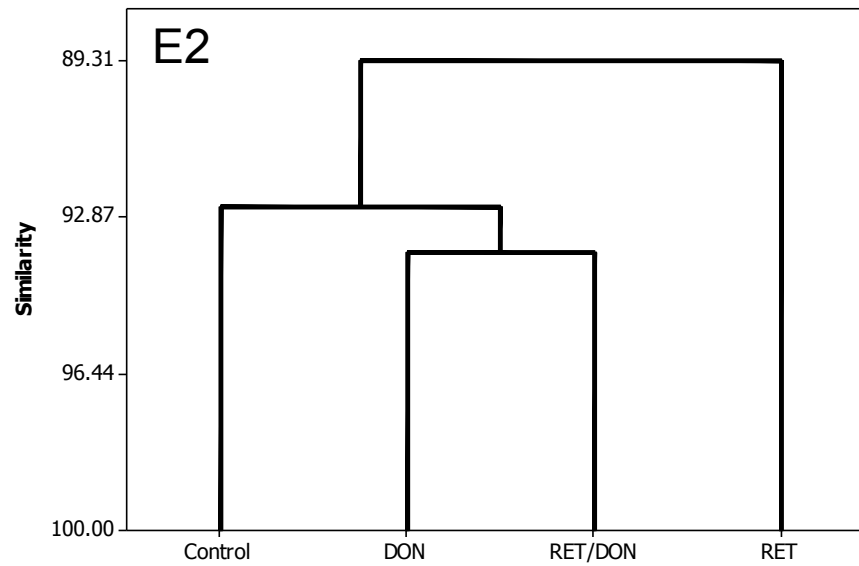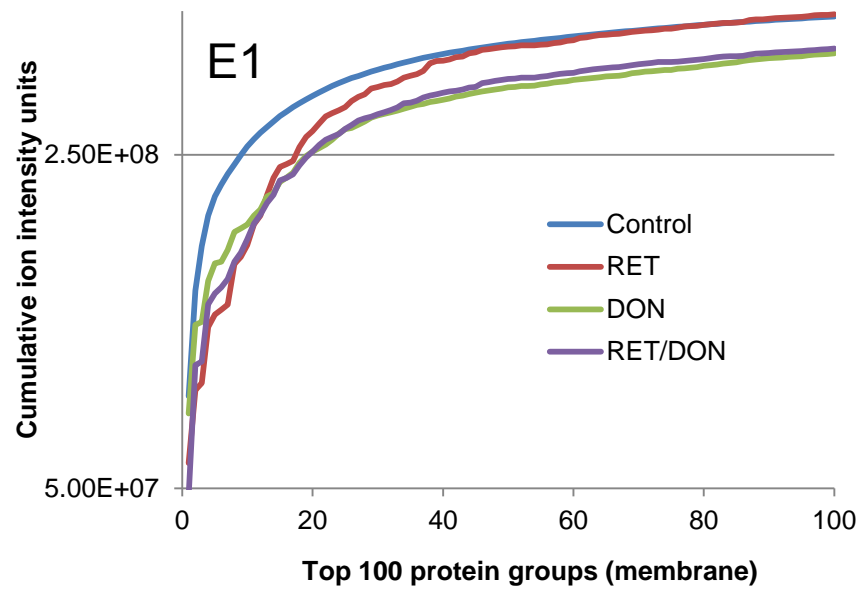
Figure 6.25. Cumulative ion intensities (1) and comparative correlation dendrogram (2) of the top 100 protein groups in different biological functions found following exposure to 10 µM DON, RET, RET and DON combined or control conditions as relative to the control. The biological functions include the control for 6 different GO-terms including A (Translational elongation), B (Protein biosynthesis), C (Apoptosis), D (Response to organic substance), E (Membrane) or F (Cell cycle regulation).

Table 6.6. Key protein groups for discussion. Green = enriched levels, yellow = equal to control levels and red = less than the control (magnification shown by gradation of colour)

	Protein group	Percentage of control ion intensity		
PEP	Gene Name	DON	RET	DON/RET
0	lactate dehydrogenase A (Anaerobic respiration)	0.2	0.1	0.1
1.99E-05	Mitogen-activated protein kinase 1 (MAPK, ROS-specific apoptosis)	105.3	98.4	123.9
1.60E-158	catenin (cadherin-associated protein), beta 1, 88kDa (cell adhesion, cancer cells)	44.4	88.1	23.5
5.17E-14	catenin (cadherin-associated protein), alpha 1, 102kDa (cell adhesion, linking actin)	40.8	106.3	25.8
4.71E-17	catenin (cadherin-associated protein), delta 1 (cell adhesion, DNA transcription)	5673.9	524.4	138.7
1.68E-06	UDP glucuronosyltransferase 2 family, polypeptide B7 (DON excretion/ detoxification)	294.2	327.5	146.1
5.27E-14	beta-1,3-glucuronyltransferase 3 (glucuronosyltransferase I) (DON excretion/detoxification)	37.8	50.1	80.6
2.06E-120	microsomal glutathione S-transferase 1 (DON/RET-specific excretion)	943.1	236.4	270.6
7.01E-06	microsomal glutathione S-transferase 2 (DON/RET-specific excretion)	145.4	100.6	113.3
3.17E-29	microsomal glutathione S-transferase 3 (DON/RET-specific excretion)	28.3	93.2	40
1.10E-06	Glutathione-S-transferase omega 1 (soluble cytosolic, DON/RET-specific, excretion)	243.9	70.8	0
1.14E-48	glutathione S-transferase kappa 1 (mitochondrial)	58.6	112.8	62.7
4.93E-09	glutathione synthetase (DON/RET-specific, glutathione synthesis)	49.5	82	26.5
1.51E-65	glutathione reductase (DON specific, oxidative stress)	83.7	173.3	478
0.000452	Cytochrome c oxidase subunit 3 (RET-specific activation enzyme)	95.1	68.3	33.6
4.29E-105	cytochrome b5 type B (outer mitochondrial membrane)	1221.0	328.6	172.0
1.89E-36	cytochrome c oxidase subunit IV isoform 1(mitochondrial)	819.3	213.4	129.7
5.82E-95	Cytochrome c oxidase subunit 2 (mitochondrial)	197.5	198.2	128.7
1.09E-21	cytochrome c oxidase subunit Vib polypeptide 1 (ubiquitous)	211.1	137.9	149.1
0.0000113	P450 (cytochrome) oxidoreductase	111.4	195.6	22.0
9.43E-13	cytochrome c oxidase subunit VIc	48.4	106.6	82.7
2.3E-83	ubiquinol-cytochrome c reductase core protein I	65.3	83.8	97.5
8.83E-06	oxidase (cytochrome c) assembly 1-like	42.0	59.5	41.9
4.72E-274	histone cluster 1, H1b	61.6	83.5	100.5
0	histone cluster 1, H1c	17.3	26.3	33.9
0	histone cluster 1, H1e	42.2	56.5	69.8
0	histone cluster 1, H2ac	280.7	206.4	100.1
0	histone cluster 1, H2ag; histone cluster 1, H2ah	3988.0	4813.2	387.3
0	histone cluster 1, H2bb	41.4	59.0	95.5
0	histone cluster 1, H2bd	0.0	5.1	17.9
8.92E-07	histone cluster 1, H2bj	46.5	69.3	43.4
1.49E-35	histone cluster 1, H2bm	108.9	183.3	158.4
0	histone cluster 1, H2bo	0.0	7.8	18.1
0	histone cluster 1, H4l	90.7	112.4	103.8
0	histone cluster 2, H2aa3; histone cluster 2, H2aa4	95.0	163.2	97.0
1.61E-78	histone cluster 2, H2ab	396.7	101.2	79.4
0	histone cluster 2, H2be	0.0	0.9	8.9
0.0050646	H1 histone family, member 0	125.8	71.5	213.1
2.9E-36	H1 histone family, member X	72.2	90.9	58.1
3.27E-16	H2A histone family, member V	2521.9	157.1	342.9
1.31E-64	H3 histone, family 3B (H3.3B)	34.0	100.9	167.5

205

6.4.12 DNA-crosslinking assays
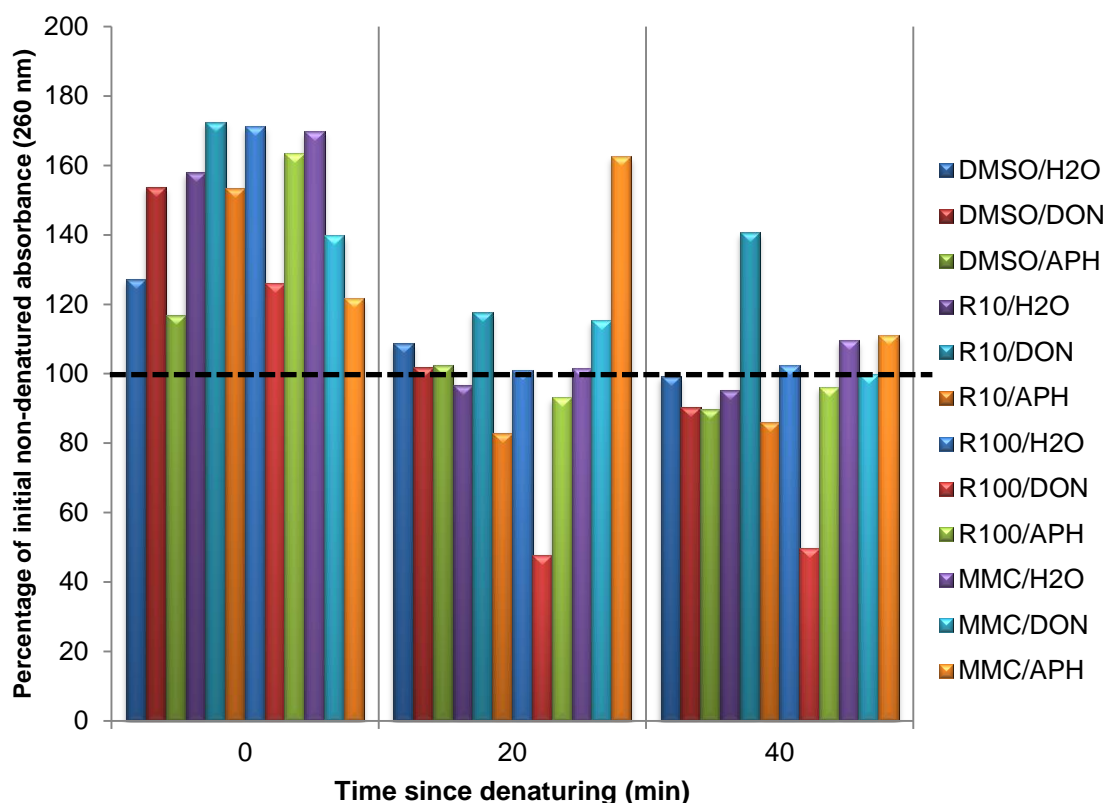
Figure 6. 26. The change in absorbance (260 nm) of HepG2 DNA following 48 h cellular exposures to combinational exposures of DON 10 µM, RET (R) 10 µM/100 µM, MMC 0.1 µM and APH 1.5 µM. Extracted DNA was subjected to denaturing at 95 °C and recovery on ice for 20 or 40 min with an increase in absorbance indicative of strand separation and a rapid annealing suggestive of crosslinking scaffolds speeding up alignment.

A preliminary UV-absorbance analysis of native, denatured and renatured extracted DNA samples suggested that heating to 95 °C and immediately determining absorbance yielded a 18-70% hyperchromatic response for all samples consistent with the separation of sister strands (Figure 6.26). As the strands realigned the absorbance in the control dropped back to initial levels, whilst there was more fluctuation in the treated, with 100 µM of RET / 10 µM of DON (the highest cytotoxic dose) producing a much lower response than the initial. This suggests the denaturing has made the DNA more organised or removed a component that initially increased absorbance at room temperature. Contrary to this, DON and RET at 10 µM yielded a reduction at 20 min followed by an increase at 40

min and MMC with APH yielded a more surprising result with annealing for 20 min resulting in a transitory 50% increase in absorbance, perhaps because the annealing has occurred too quickly and the strands are not aligned properly. By using a different aliquot of the same DNA to undertake the same denaturing process, but this time running the sample on an agarose gel, it was possible to observe the different fragment sizes making up the native and denatured fractions (Figure 6.27.). The first observation is the value of the DNA markers to provide a positive control for denaturing and an idea of the differential distance single stranded fragments migrate compared with non-denatured DNA, for example it is not a proportional relationship with 250bp actually migrating faster in duplex form.   The second observation is the different array of bands/smears in terms of position and intensity for each non-denatured lane. This illustrates the difficulty of undertaking a study with crosslinked genomic DNA where normalisation between samples using spectrophotometry becomes troublesome due to the different size trends present in the sample. It would have been simpler to use a uniform fragment size (i. e. a single band from a plasmid) to evaluate crosslinking and annealing dynamics.

RET treated DNA is retained in the well with denaturation bands tending to disappear rather than appear as the single stranded form indicating unrepaired labile nucleotide excision repair gaps along the strand were prevalent leading to a range of small single stranded DNA fragments that migrate to leave a smear rather than distinct bands. The mechanism is thus similar to the comet assay in that a labile or excised base leaving a gap leads to a strand break on denaturation. The total amount of DNA remaining in the well seems to decrease on denaturation illustrating at least a proportion is only retained due to inter- and intra-stand DNA and protein linkages.   A dose of 10 µM DON results in very

little genomic DNA remaining in the well indicating that karyolysis/karyorrhexis has already occurred in the majority of cells resulting in fragments of a range of shorter sizes do not form a band.
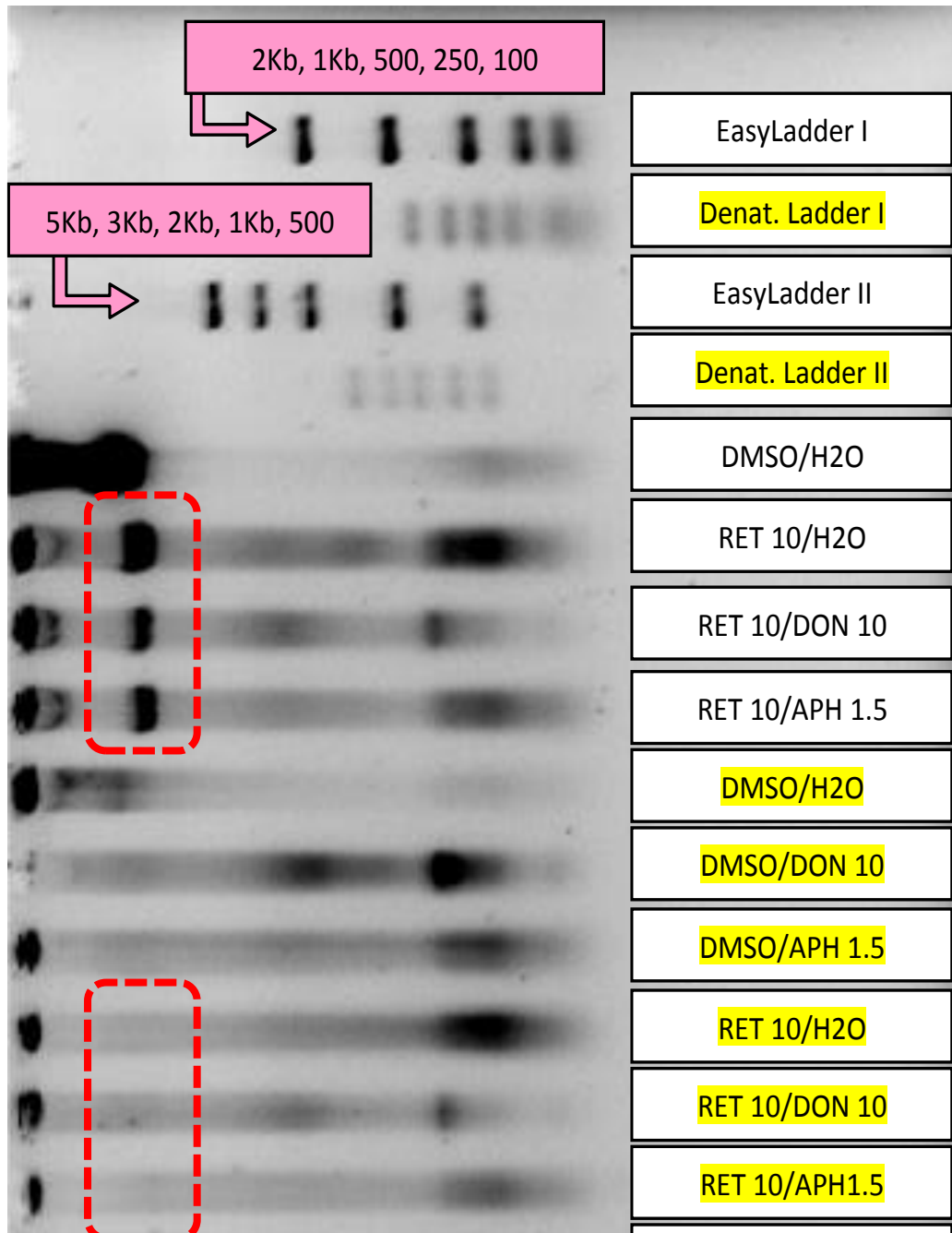
Figure 6.27. DNA crosslinking assay comparing the migration pattern of denatured and normal DNA extracted from HepG2 cells exposed to different combinations of DON (10 µM), RET (10 µM) and APH (1.5 µM) 48 h. DNA from each treatment was normalised to 100 µg/ml, denatured at 95 °C for 10 min prior to 25 µl transferred into a 1% agarose

gel for electrophoresis (labelled yellow) with DNA ladders used as a positive control for denaturation

6.4.13 Results Synopsis

Table 6.7. Summary of all cytotoxicity and cellular viability assays with GLM regression statistics. The main effects attributed to DON or RET or an interaction is given   The individual cell data was used for regression in the comet assay where wide variation and clustering requires interrogation of raw data, hence the contribution to R-Sq values are low.

Assay	Measurable endpoint	DON main effects	Retrorsine main effects	RET/DON interaction	DF	R-square	Description
Comet	DNA crosslinking/ strand breaks	1.2%, F = 24.32, $p< 0.001$	4.0%, F = 70.96, $p<0.001$	4.2%, F=24.32, $p< 0.001$	4799	9.3%*	Dome response to RET reduced by exposure to DON.
Micronucleus	Mitotic aberrations, chromosome and fragment loss	25.3%, F = 4.08, p=0.008	6.23%, F = 1.37, P =0.266	12.69%, F = 0.68, p =0.757	55	44.2%	Increase in micronuclei with DON. RET in isolation yields a dome response.
NDI	Rate of cell proliferation	59.2%, F = 15.25, $p<0.001$	1.5%, F = 0.52, p =0.674	4.4%, F = 0.38, p =0.963	55	65.1%	Inhibition of cellular proliferation significantly linked to DON concentration only
MTT	Mitochondrial viability	46.0%, F = 31.12, $p<0.001$	10.9%, F = 7.35, $p<0.001$	3.8%, F = 0.84, p =0.579	95	60.6%	DON significantly reduces mitochondrial vitality whilst RET actually reverses it
Carboxy-fluoroscein-diacetate metabolism/ propidium iodide uptake	Mitochondrial metabolism and cellular membrane integrity	22.1%, F = 3.52, p =0.024	1.4%,F = 0.34, p =0.716	1.2%, F = 0.09, p =0.996	47	24.7%	DON concentration major contributor to mitochondrial/membrane compromise
48 h cell density (mm²)	Rate of cell proliferation	60.5%, F = 17.4, $p<0.001$	1.5%, F = 0.58, p =0.634	3.2%, F = 0.31, p =0.984	59	65.2%	Inhibition of cellular proliferation significantly linked to DON concentration only
Trypan blue exclusion	Loss of membrane integrity	63.0%, F = 19.59, $p<0.001$	1.3%, F = 0.52, p =0.671	3.5%, F = 0.37, p =0.968	59	67.8%	Membrane integrity significantly linked to DON concentration only
Total cellular protein	Rate of cell proliferation	71.5%, F = 47.14, $p<0.001$	6.7%, F = 4.47, p =0.010	5.6%, F = 1.23, p =0.314	47	83.8%	Inhibition of cellular proliferation significantly linked to DON concentration only
Proteomics	Determination of differences in protein group enrichment	DON and RET/DON produced the greatest expression of proteins associated with apoptosis. DON was found to effect protein synthesis in two different ways; by reducing the protein groups related to protein synthesis as a whole, but increasing the proteins related to translational elongation. DON induced the most 4 fold protein group increases and decreases compared to the control.					
Crosslinking assay	Determining the degree of crosslinking of DNA	Crosslinking adducts yield breaks and increase in fragmentation rather than acting as scaffolds keeping sister strands attached. The possibility that protein crosslinks make up a significant portion of the lesions is suggested. DON (10 µM) produces DNA which is already undergoing karyorrhexis.					

6.5 Discussion

The current investigation focuses on the genotoxicity and cytotoxicity of RET and DON exposed singly and in combination to a human liver cell line; HepG2. In addition to a suite of different assay endpoints, the study incorporates positive controls for crosslinking and inhibiting repair of DNA, to aid in toxicity mechanism characterisation. Analysis of DNA crosslink stability and proteome enrichment further describes the effect of these toxins on exposed cells, although the data collected suggests the relationship between these cytostatically potent dietary toxins is far from simple and requires tentative interpretation.

6.5.1 DNA and protein crosslinking

RET was found to exert a dome-like $\log_{10}$ dose dependant genotoxic response in both the comet and MN assays. Low doses increased tail length and MN frequency indicating presence of strand lesions, whilst a dose of 100 μM yielded a reduction suggesting excessive crosslinking. This reduces the potential for strand separation and fragment migration in the comet assay and initiation of mitosis in the MN assay, which is in agreement with other HepG2 toxicological studies (Kevekordes *et al.*, 2001). The cell cycle has a significant influence on comet assay data and it is possible that as DON reduces cellular turnover the dome-like RET response is reduced as the cells are essentially stalled in interphase by DON and any accumulated damage can be repaired (Tiemann *et al.*, 2003), including labile sites formed by a single alkylation at low doses or migration-limiting DNA/protein crosslinks at the highest RET dose.

Work by Kim *et al.* (1995) suggests DNA-protein crosslinks make up around 50% of the total crosslinks in the cell following exposure to the pyrrole forming PAs, of which a large proportion are actin-DNA crosslinks, presumably acting as chromatin scaffold proteins in

the nucleus and consequently being adjacent to the DNA. In the current study, PA-exposed cellular DNA was run on an agarose gel with and without denaturing (95 °C). It was highlighted that several bands disappeared completely compared with the native DNA. If it was cross-linked duplex DNA, it would have been unlikely that the sister strands would irreversibly separate according to work by Tang and colleagues (2008), and when comparing the single strand and duplex ladders it would have been expected that the same key band would have reappeared a short distance down the gel. It seems sensible to hypothesise that the key band is in fact a DNA-actin crosslink that on denaturing is no longer visible, and it is an oversight that an immuno-blotting technique or proteinase-K comet was not employed at the time to confirm this, as it could aid in explaining some of the complex genotoxicity results. DNA-protein crosslinks can be seriously detrimental to cells in that they are bulky DNA adducts which inhibit DNA metabolism leading to mitotic arrest and the existence of megalocytosis (Kim *et al.*, 1993). Since the candidate proteins are likely nuclear matrix associated proteins such as actin, which are intrinsic to DNA replication, transcription and repair processes (Miller *et al.*, 1991), it could be twice as disruptive to nuclear and cellular function. The increase in nucleoplasmic aberrations observed with RET treatment in the micronucleus assay could be linked to this phenomenon. It must be remembered that following a 48 h exposure to the toxins there is a 24 h incubation with the MT cytochalasin-B, an actin polymerisation inhibitor that could disrupt the cytoskeleton function yet further (MacLean-Fletcher and Pollard, 1980).

When considering the use of the CBMN assay with interacting genotoxins and cytostatics, it needs to be considered that only micronuclei associated with BNC are scored to prevent confounding effects caused by suboptimal cell division kinetics, a factor that could be

significantly important when the two mechanisms are concurrent. Logistically, the reduction in the proportion of BNC due to reduced nuclear division makes the scoring process longer. This suggests that the only cells exhibiting genotoxicity are those able to divide unhindered and not damaged sufficiently to undertake apoptosis. This is exacerbated by the addition of Cytochalasin-B which inhibits cytokinesis by interacting with actin polymerisation. If a cytostatic is tested in the CBMN test it is impossible to exclude the interaction of this mechanism with cytochalasin-B, as although rinsing with PBS prevents carry over, the modification to cell cycle kinetics/protein enrichment could influence cytokinesis.

One theory that could be applied to the antagonistic response of DON to DNA damage and crosslinking by RET is the element of recovery time. As DON reduces cellular turnover (especially at 0.1 µM), the time required to repair lesions in increased resulting in less migration in the comet assay and less chromatin loss in the CBMN assay. However, there is a point where a DON concentration threshold is reached and apoptosis is prevalent (10 µM), or with both toxins (10 + 10 µM) when apoptosis is increased further by 25% and there is additional disruption to the cell cycle proteome leading to cellular senescence and megalocytosis. The balance between DON-associated apoptotic induction and RET-related cellular senescence governs the latent effects of acute toxin exposure. Hincks *et al.* (1991) found cells exposed to RET remained megalocytotic / senescent for a long period of time without undergoing apoptosis. This is notably mirrored *in vivo* where chronic PA exposure causes unrepairable and accumulative damage in the liver leading to VOD and cirrhosis in livestock (Molyneux *et al.*, 1988) and humans (Kakar *et al.*, 2010). If same cells had undergone apoptosis, the harmful

chronic accumulation of megalocytosis would be avoided along with the risk of carcinogenesis.

The current investigation employed positive controls; APH as a DNA-repair and replication inhibitor, MMC as a potent DNA-crosslinker, to validate the assays whilst providing additional indices of toxicological interaction. APH not only inhibits DNA repair by interacting with at least three of the mammalian polymerases, it also prevents DNA replication and therefore nuclear division (Levenson and Hamlin, 1993) and yields double strand breaks (Kurose *et al.*, 2006). The accumulation of incision sites following agent exposure with the addition of APH has been proposed to increase the sensitivity of genotoxicity assays (Speit and Schütz, 2008). The current results with APH treatments however indicate that reductions in cellular turnover, membrane integrity and esterase activity were more pronounced than any noticeable increases in accumulated DNA damage, indicating that the cells may be stalled in one stage of the cell cycle.

Apoptotic processes are intrinsically linked to cell cycle progression checkpoints and arrest. In fact, the stage of the cell cycle exposed can determine the susceptibility of the cell to certain toxins; including DNA replication inhibitors and crosslinking agents. For example, APH was found by Kurose *et al.* (2006) to be most inhibitive during S-phase (actively replicating) and ineffective during the G_1 (Initiation of cell division, non-replicating) and G_2 (DNA repair, commitment to mitosis, non-replicating) checkpoint gap phases. Furthermore, cells may be already stalled in these non-replicating phases due to a binary exposure with another toxin, RET for example, therefore cell cycle phase-specific toxicity could be altered. Taking this further, a cell arrested in G_2 would have a larger volume due to the doubling of DNA and enrichment of enzymes and metabolic pre-

cursors required post-cytokinesis. Consequently, cells stalled at this stage may have a greater individual capacity for metabolism of xenobiotics (i. e. MTT, CFDA) due to the increased presence of specific enzymes and comet tail length could be increased by the presence of double the amount of DNA. Likewise, arresting cells in G_2 enables the repair of mitotic aberrations, which can reduce damage measured in the CBMN assay with a reduction in cell turnover. This can be considered another level of complexity regarding interactive toxicity that needs to be considered

MMC yielded robust crosslinking responses in the genotoxicity assays, which aided in explaining the dome-like RET response. The effect on cellular proliferation and DNA fragmentation of a slightly crosslinking (0.1 µM) and lethally crosslinking (10 µM) dose was clear, although cellular esterase activity was reduced whilst protein levels were strangely increased, perhaps driven by a stress response or the cell cycle effects mentioned above. DON and MMC co-exposure yielded a broadly similar interactive response to DON and RET.

6.5.2 Oxidative stress and apoptosis induction
A dose related increase in DON-associated aberrations scored in the micronucleus assay may be explained by the increased prevalence of cytome phenomena such as nucleoplasmic budding and bridging, caused by disrupted nuclear membrane and cytoskeleton integrity rather than direct DNA damage-yielding micronuclei, an observation also recorded by Yang and co-authors (2014) with human lymphocytes. The same group suggested that any DNA damage stemming from DON exposure was due to oxidative stress due to depletion of mitochondrial cytochrome-c rather than direct interaction. This fits well with the proteomic data in the current study where such

enzyme groups were upregulated with DON to presumably compensate for reduced ROS depletion capacity. Li and co-workers (2014) found DON to induce effects in chicken embryo cells including mitochondrial membrane disruption, apoptosis and disruption of the cell cycle. Zhang and co-workers (2009) exposed HepG2 cells to DON over a 1 h period with and without an antioxidant and concluded that the DNA damage recorded when exposed to 3.75-30 μM DON was due to ROS, although significant increases in ROS measurement were only found at a higher dose of 60 μM. DON was however not found to increase DNA migration in the current study and instead a prevalence of apoptosis is recorded, which could be due to the 48 h incubation permitting initiation of apoptosis (yielding un-scorable cloud-like nucleoids), whereas a shorter exposure would observe an increase in oxidative DNA lesions. Key research has suggested plasma membrane damage is less encountered than late stage apoptosis and DNA damage in regards to DON cytotoxicity (Minervini *et al.*, 2004). This agrees well with the current findings where trypan blue results are not as conclusive as apoptosis and cellular turnover assays. The progenitor cause of this oxidative stress could be the downstream effects of DON-ribotoxicity; the inhibition of peptide elongation in the ribosomes, specifically those required for enzymes used in the modulation of ROS concentration. Pan and co-workers (2013a) published an extensive proteomic report on the early stage phosphorylation dynamics of acute DON-induced ribotoxicity in mice splenocytes, and found initialisation of MAPK and PI3K/AKT intracellular cascade pathways controlling intracellular signalling, cytoskeletal processes, apoptosis regulation and nucleus organisation. The current work however focusses on late stage protein ontology characterisation after such cascading would have occurred and the effects of stalled ribosomal components, reduced protein synthesis and late stage apoptosis and cellular senescence is instead a possibility.

6.5.3 Metabolism and protein enrichment

Whilst the assay endpoints in the current study focus on tangible decreases in cell vitality or increases in DNA instability, it is worth considering how the metabolic status of the cell could be affected by DON-induced ribotoxicity. A reduction in phase I activation enzymes would augment reduced metabolism of RET to its deleterious nucleophilic pyrrole form, resulting in less DNA damage. Likewise, a reduction in phase II excretion would yield an increased RET potential.

Chronic low levels of DON in mice have been linked to a reduced xenobiotic metabolising capacity in terms of glutathione conjugation of trichothecenes, yet P450 enzymes were unaffected (Gouze *et al.*, 2006). T-2 toxin, another potent *Fusarium* MT which also shares the same protein synthesis inhibitory toxicity mechanism as DON, did not affect drug metabolism in Rabbit livers *in vivo* when exposed through the dietary route (Guerre *et al.*, 2000). T-2 did conversely increase the activity of phase I and II enzymes in piglets administered sub-cutaneously (Dong *et al.*, 2008), significantly for 24 h and less so for the following 48 h, but the levels were reduced again at 48 h. Xenobiotic enzymes found with western blotting to be upregulated included: CYP1A2, CYP2E1, CYP3A4, glutathione-s-transferase and GST M1-1 (as was found with DON in the proteomic analysis). It would therefore appear DON exposure would not likely reduce the amount of RET being metabolically activated, but it would reduce the potential for excretion of the DNA-reactive pyrrole intermediate by the glutathione-s-transferase route as glutathione levels are depleted (Fu *et al.*, 2002). Since both toxins were exposed concurrently it can be assumed that such a depletion relationship is not as pivotal, since both compounds are exposed to the same initial cellular glutathione concentration (assuming equal passage through the cell membrane). When the concentration of one toxin is increased 10-fold

and the other remains at the same concentration it could be hypothesised that there would be less detoxification potential proportionally, thus increasing the concentration of DON may yield more pyrrole adducts indirectly (Yan and Huxtable, 1995). It can be added that equal competition for binding sites is reliant on DON or RET having a similar affinity for the enzyme involved. Strange and Hayes (2000) noted that there is a large degree of redundancy in regards to substrate detoxification in microsomal and cytosolic glutathione families. A similar amount of metabolic redundancy can be found with the cytosolic hydrolysis of carboxyfluorescein-diacetate in the CFDA assay. This metabolism can be accomplished by a multitude of proteases, lipases and esterases within the cell, and the degree of de-acetylation is a proxy for cellular vitality (Kessel *et al.*, 1994). DON at the lowest tested concentration (0.1 µM) was found to decrease cellular proliferation (as measured by the protein, cell count and MTT assays), yet was found to increase the hydrolysis of CFDA, especially if combined with RET or MMC. One hypothesis is that as the cells divide at a reduced rate due to ribotoxic stress / DNA lesions, the cells have more time and resources to up-regulate the proteins needed for maintaining homeostasis, including enzymes required for CFDA metabolism. Though there are fewer cells, providing apoptosis has not been induced, they are individually more metabolically active. This phenomenon could be associated with the arresting of the cell cycle in G_2 phase as discussed earlier, *i. e.* the cells have more facility for metabolism of CFDA due to the presence of post-mitosis daughter cell levels of enzymes in pre-mitosis single cells.

Interestingly, the esterase activity could also be linked to the susceptibility of the cell to RET exposure. Two main routes to PA metabolism are present in the liver. Cytochrome P450 produces the crosslinking pyrrole form whilst a metabolism of the RET with

esterases produces a low toxicity molecule which can be more easily excreted (Prakash *et al.*, 1999b). In rats with depleted esterase activity it has been shown that a larger proportion of the toxic pyrrole was produced than the non-toxic necines and necic acids (Mattocks, 1981). The metabolic status of the HepG2 cell line dictates which metabolites and hence which effects may be induced and whether these could be modulated by co-exposure with DON is still unknown.

6.5.4 Critical evaluation of interactive toxicity strategy and use of HepG2 as surrogate model for mammalian RET/DON toxicity

This study is in effect a pilot as there is very little in the literature evaluating the interactive toxicity of a crosslinking agent and protein synthesis inhibitor. Whilst this may be perceived as a gap in scientific knowledge ripe for further assessment, the results presented in this chapter highlight the reason for such a research opening! Toxicological analysis of mixture data is highly intricate and convoluted that demands a large degree of scoping to tease out relevant interactive relationships worth pursuing further. Only then can a robust interactive strategy be undertaken. The evaluation of multi-MT toxicological interactions has been undertaken with different methodologies with some focussing on mathematical models derived from isobologram techniques (Alassane-Kpembi *et al.*, 2013) whilst others adopt a more direct statistical comparison approach (Lei *et al.*, 2013). The problem exists when both antagonistic and synergistic toxicity is present concurrently over a dose-response curve. The current results, in regards to cytotoxicity, indicate RET to be antagonistic at low doses and only synergistic/additive at 100 µM. DON has a more linear dose response and is the principle cytotoxic component recorded when in combination with RET. The use of a wider range of concentrations and time course sampling strategies would greatly benefit this study, especially when trying to pin-point

the threshold interactive dose when cellular proliferation is reduced. With so many facets of cellular toxicity that could be targeted with RET / DON exposure it can get overly complex, although scoping for key mechanisms is an important part of toxicological investigation. It is perhaps more effective to focus on just cellular proliferation as a single endpoint, since it is a shared effect of both toxins and nests together the latent effects of geno- and cytotoxicity. Such an approach was utilised by Alassane-Kpembi and co-workers (2013) who used lysosomal integrity as the sole endpoint when considering binary and ternary trichothecene exposures, and therefore could generate more robust indices of interaction but without the mechanistic evaluation.

In terms of the proteomic characterisation, it must be considered that the HepG2 cell-line is derived from a human liver tumour and as such, the degree to which the results can be extrapolated to *in vivo* scenario needs to be carefully considered. Some workers have noted that HepG2 cells do not have the full phase I and II enzyme complement for toxin metabolism compared with primary hepatocytes which are more efficient at bio-activation of pro-mutagens (Wilkening *et al.*, 2003). Others have described HepG2 cells as having one of the better metabolic competencies for an established cell line (Roe *et al.*, 1993, Kevekordes *et al.*, 2001). This negates the use of rat liver S9 fraction, which actually yields inferior metabolic activation of PAs to primary hepatocytes in any case (Müller *et al.*, 1992). This can be further improved by utilising 3D culturing techniques (such as spheroid culture), to restore native liver-like metabolic activity (Xu *et al.*, 2003). In addition, HepG2 cells were shown to have similar glucuronidation activity to freshly isolated human hepatocytes (Grant *et al.*, 1988), and thus have a mechanism for DON and RET excretion *in vitro* (He *et al.*, 2010b), which non-liver cell lines lack (UDP-

glucuronosyltransferase was found enriched in the DON, RET and binary exposure in the current study).

Extrapolation of the *in vitro* toxicological results collated in the current study to an assessment of human and production animal risk is not possible without additional *in vivo* experiments. *In vitro* data does not account for differential rates of uptake during a dietary exposure (due to intestinal integrity and gut flora, (Akbari *et al.*, 2014)) or the inter-individual variation in metabolism (due to body size, hepatic blood flow, liver weight and reserve capacity, age, sex and genetics, (Rostami-Hodjegan and Tucker, 2007)). However, the genotoxicological and cytological information gathered *in vitro* could help design a robust and efficient *in vivo* experiment in relation to which parameters to focus on. It would be interesting to obtain acute and chronic toxicokinetic data of both toxins individually and together, for DON has been shown to effect gut wall morphology and intestinal membrane functionality enabling increased uptake of xenobiotics (*viz.* RET) in mice (Akbari *et al.*, 2014) and chickens (Osselaere *et al.*, 2013). Since DON exerts toxicity in both liver and intestines, it would be again difficult to distinguish the exact mode of interactive toxicity with RET. Measuring bulk reductions in animal growth rate and increases in liver malignancies and histopathological lesions would confirm whether a pertinent problem exists with co-exposure of DON with RET, and whether the cell cycle and genotoxicity effects observed *in vitro* were actually valid biomarkers of effect. It would be interesting to determine if chronic exposure to DON predisposes the liver to increased uptake, metabolism and damage by RET as this would be a first for any DON-interaction study.

6.5.5 Concluding remarks

Whilst cellular exposure to RET results in predominantly DNA and protein crosslinks that hinder cellular proliferation (Li *et al.*, 2013), DON yields a cytotoxic response based on ribotoxicity (Pan *et al.*, 2013b), ROS production and apoptosis, with DNA damage induced indirectly (Bony *et al.*, 2006). The toxicological interaction between these two hepatotoxins is highly complex with the exact mechanisms remaining unclear, perhaps due to each toxin individually targeting multiple sites within the cell, differential effects on the cell cycle and multiple metabolic effects (Carpenter *et al.*, 1998). Further work is required using a single assay for simplicity, but with multiple time-points and a focus on toxins exposed over longer time scales at chronic concentrations. In the greater scheme of toxicological burden associated with natural toxins, the use of models to predict toxicity profiles derived from mixtures of compounds would be useful for legislators working to improve food/feed safety. However, the results highlighted in the current investigation suggest mechanistically that interactive toxicity is not a simple task of additive impact, but often a combination of a mixture of additive, synergistic and antagonistic responses over a range of dose combinations. This is supported by results collected by Mckean and co-workers (2006) working with binary AFB_1 and Fumonisin B_1 toxicity in HepG2 cells which suggests combinational toxicity follows a bi-phasic pattern, with chronic doses often antagonistic and acute doses yielding additivity. Collation of structural indicators of toxicity and known toxicological mechanisms, tissue specificity and cellular targets is paramount when evaluating toxicity risk associated with co-exposed toxins. The health implications of multiple feed and food-borne toxin exposures should not be under estimated or ignored.

7 General discussion: DON toxicity and biological remediation strategies, a perspective.

This study explores many facets of DON research; from DON-contaminated spikelets to liver cell proteomics. It has been shown that DON is extremely potent in terms of toxicity, resistant to the effects of pH in fermented feeds and is unaffected by the presence of LAB isolated from birds or commercially available LAB silage additives. However, there is an exciting suggestion that this robust molecule could be removed from contaminated CG by resident phyllosphere LAB under specific ensiling conditions.

The original research question fielded in the initial stages of this investigation was whether LAB from liquid feed, commercial fermented feed inoculants or LAB from wild birds could play a role in DON removal in liquid media. It was swiftly noticed that this was not to be. Knowing when to pursue an anomalous result further and when to change tack and develop new hypotheses is well illustrated in the current thesis. It was initially envisaged that a LAB would be able to degrade DON and its degradation products could be identified and tested for toxicity in an *in vitro* cellular experiment (fortuitously this was exactly what was possible with the equally as toxic, yet more labile patulin (Appendix 10.2). The scheme of events with the current DON investigation was too reliant on a positive result, hence the change of tack to CG feeds, a novel area in regards to MT research. While published studies on CG are few in number (11 articles from 1950 to present in Web of Science when using "crimped grain" as keywords (WoK, 2015)), the application itself has a multitude of agricultural benefits such as: shorter cropping cycles, higher nutritional value and on-farm feed traceability.

Following the sourcing of freshly harvested CG for a spike and recovery experiment, 3 silos from 30 were found to have a significant reduction in DON, and all 3 were heat treated to reduce the microbial community prior to addition of DON. At the time of the first CG experiment there was only a short window between collecting the CG and ensiling. CG has a relatively limited functional lifespan as once crimped, it can rapidly deteriorate through oxidation and hydrolysis to lose its realistic feed properties. With the benefit of hindsight the mechanism of heat treatment would have been further characterised at this point, for it is unclear whether thermal inactivation of resident inocula or modifications to the CG matrix (*i. e.* anaerobicity, nutrient availability, water activity) were chiefly responsible for the shift in microbial assemblage and whether this augmented a biological reduction in DON, or conversely the heat treatment produced a matrix change which physically or chemically interacted with DON directly.

Whilst there was a drop in DON recovery with the heat treated grain, without definitive evidence of metabolism, such as the identification of novel peaks in the HPLC-UV analysis, the detoxification mechanism remains unknown. It is possible that a "dilute and shoot" LC-MS/MS approach would be more fruitful in determining if any specific DON metabolites were present at all using the selected-ion monitoring (SIM) approach without the requirement for immunoaffinity clean-up, and hence without the structural bias of DON-specific antibodies or effect of pH (Malachová *et al.*, 2014). The SIM approach was extremely successful with identifying the PAT metabolites in the attached hydroascladiol paper (Hawar *et al.*, 2013), although it helps if the parent molecule and MS fragments M/Z are already known or mathematically derived. Unfortunately, the decrease in DON *in vitro* with *W. cibaria* and *H. alvei* did not translate to an adequate decrease under silo

conditions with naturally contaminated grain. Should more work be undertaken with these bacteria it is important to heed the recommendations by Jard *et al.* (2011) in order decide if they are viable degradation agents. The biotransformation must be swift and the bacteria should be able to undertake the reaction under a range of oxygen, nutrient, water activity and pH conditions. The biotransformation products need to be assessed for toxicity *in vivo*. The bacteria need to be non-pathogenic and must not express antibiotic resistance. A further beneficial trait would be the LAB possessing the enzymatic capacity for hydrolysis of D3G to DON in order effect a more thorough DON removal in the grain. Berthiller *et al.* (2011) found *L. plantarum* could hydrolyse 62% of D3G in 8 h at 37 °C in liquid media compared with other LAB which were negative for this property.

Although the primary function would be to degrade DON, it would be worth testing the LAB as a potential BCA in the field or as a silage additive in isolation using the mould spore inhibition assay and by measuring solid state fermentation parameters further. *W. cibaria* has already shown a reasonable ability to produce acetic and lactic acids, although whether it can compete with homolactic *Lactobacilli* in naturally colonised grain is currently unknown, although *W. cibaria* has been found to dominate maize silage under certain conditions (Pang *et al.*, 2011). The additional ability to degrade PAs, such as RET, within the silo would also reduce the toxicity burden of co-occurring silage toxins in cattle TMR. This would be well received by the dairy industry considering the accumulative PA liver toxicity (Craig *et al.*, 1991) and carryover of PAs into milk (Hoogenboom *et al.*, 2011). Incidentally, the research on PA detoxification in silage is rather limited, notwithstanding one article by Candrian and co-workers (1984), who recorded a significant metabolism of PAs with grass silage compared with hay.

Decreasing DON or PA levels in silage would mean little if the resulting feed was inferior nutritionally, unpalatable and reduced animal productivity. It must equally be considered that the nutritional components of the feed including; antioxidants, micronutrients, and probiotic LAB, may impart a protective role against DON in their own right. Furthermore, if the mechanism of DON reduction in silage was through binding, it would be worthless if the bond was insufficiently strong to pass through the GI tract intact and would be considered "masking" the actual toxicity of the feed. The same could be said for biotransformation where the end-products may be equally toxic, but may escape detection by IAC-RP-HPLC due to structural chemical changes.

There is little doubt that reducing the amount of toxigenic *Fusarium* sp. in a crop to begin with is a better overall strategy than removing the MT's postharvest. However, climatic anomalies (Magan *et al.*, 2011) and an imminent EU restriction on azole fungicides (European-Commision, 2011) may nullify any previously *Fusarium*-limiting farming practices, such as those discussed previously by Edwards (2004). Some growing seasons yield an unusually high DON contamination, such as in the USA in 1993 when over $2 billion was lost on the value of wheat and barley due to DON levels, on top of the yield losses caused by FHB itself (McMullen *et al.*, 1997). Had the grain been salvaged with a biological agent in a fermented feed context, then it is possible that the financial loss could have been lessened. Another avenue of interest would involve applying a BCA in the field as a prophylactic additive to outcompete and inhibit *Fusarium* growth and ideally degrade DON immediately following production therefore preventing accumulation in the grain. The use of BCAs rather than fungicides is a major development area, especially in organic farming (European-Commision, 2014). An added benefit would be that should

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the grain be ensiled, then the BCA at a sufficiently high seeding density could continue to reduce DON within the silo. This would be the ideal situation with *W. cibaria,* as it was isolated from the phyllosphere of barley initially and would benefit from a higher seeding density to shape the initial community assemblage and effect DON removal, on the proviso that results under laboratory conditions can be transferred effectively into the field conditions.

The banning of azole fungicides, whilst reducing the burden of manmade xenobiotics in food and feed may indirectly increase the amount of MT entering the food/feed chains. There can be no question as to the potency of many of the trichothecenes MTs and this should be weighed up against the toxicological risk of existing fungicides to the producer and consumer. It is also worth mentioning that in addition to fungicides, there is a EC-led decrease in the number plant protection products available for controlling toxigenic weed species in arable crops and pasture (e.g. Atrazine (European-Commision, 2011)), with a speculated knock-on effect being an increase in the amount of PAs such as RET entering the feed chain. This process of balancing up the overall human and environmental toxicological burden of food and feed in terms of manmade and natural co-contaminants is a current research direction for eminent ecotoxicological scientists such as Dana Kolpin (Kolpin *et al.*, 2010) and is a research question currently being investigated in our laboratory using a zebrafish embryo development assay. This field can be aligned with a growing public awareness of climate change, heavy metal burden, persistent organic pollutants, endocrine disruptors, soil loss, food miles, food safety and food security. A holistic view of these areas of human impact against a growing agricultural need is required, especially when considering the human population is predicted to surpass 9.6

billion by 2050 (UN, 2013). Every aspect of agriculture will be scrutinised for nutritive losses, including CG feeds; therefore more research is required in this area.

In harmony with a growing interest in the risk from grain-borne MT toxicity is the increased research into the effects of MT mixtures (Tatay *et al.*, 2014, Ruiz *et al.*, 2011a, Ruiz *et al.*, 2011b, Prosperini *et al.*, 2014). It has always been appreciated that MT's seldom appear individually within a commodity. Trichothecene MTs such as DON, 3AcDON, ZEA and T2-toxin co-occur in grain leading to co-exposure in the consumer (Ibáñez-Vea *et al.*, 2012). Furthermore, grain can be contaminated with a range of MT/phytotoxins which are equally as hazardous as trichothecenes, yet little attention has been made to determine whether the co-exposure of the omnipresent DON with non-trichothecenes yields notable synergistic toxicity. The co-exposure of AFB_1 (*A. flavus,* cancer initiator) with Fumonisin B_2 (*F. verticillioides,* cancer promoter), both found to co-occur in maize and intrinsically linked to increased incidences of hepatic tumorigenesis in rats, humans and rainbow trout (Gelderblom *et al.*, 2002b) may be noted as the driver for many MT interaction studies, yet it must also be appreciated that Hepatitis C can also synergistically interact with AFB_1 to increase the likelihood of liver cancer (Kew, 2003). Whether DON plays a similar role as a cancer promoter has not yet been explored, although it is ubiquitous enough in a wide range of commodities to warrant further studies. However, the results *in vitro* presented here do not entertain this possibility of such a relationship with RET, a less potent carcinogen than AFB_1.

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Table 7.1. The systems approach to counteracting MT contamination as introduced at the 8th World Mycotoxin Forum (WMF, 2014). The research areas which are related to the current study are highlighted with an asterisk.

Contributory factors / preventative agricultural methods	Research led MT prevention strategies	MT analysis	Storage/processing methods	Food safety and food security
Weather conditions*	Breeding	Sampling*	Storage*	Human health risks*
Climate change	Genomics	Analysis*	Processing*	Animal health risks*
Land use	Varieties	Metabolomics	Safe decontamination*	Food and feed safety*
Crop management*	Bio-control*		Transport	Food security*
Good agricultural practice*				Regulations

Whilst a lot of research has been undertaken, the toxicological and financial effects of DON continue to occur. The challenge remains to exclude DON from food and feed, yet there is unlikely to be a one size fits all remedy any time soon. Any significant reduction in DON impact is more likely to due to an integrated response based on the systems approach encompassing HACCP (hazard analysis and critical control points) and endorsed by the FAO and later updated at the World Mycotoxin Forum, 2014, in Table 8.1 (WMF, 2014, FAO, 2001). This study is aligned to many of the research areas outlined and whilst scoping in nature, the information gathered is hopefully a springboard for further work on DON remediation in fermented feeds.

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9 Appendix

10.1 Additional figures and tables

Table 10.1. Enrichment of protein groups compared with the control for HepG2 cells exposed to 10 µM DON, RET or both for 48 h. Each protein group is shown with the PEP value which is the significance probability of the identification ranging from <0.001 as a minimum to zero (absolute identification). The minimum percentage increase to be considered enrichment was 4-fold (400%) or above. Functionality of each protein is grouped as previously and labelled as follows: PS (protein synthesis); Tr (translational

elongation), Apop (apoptosis); Mem (membrane); ResOS (response to organic substance) and CCR (cell cycle regulation).

PEP	Gene Name	DON	Percentage of control ion intensity
4.71E-17	catenin (cadherin-associated protein), delta 1 (*Mem)*		5673.9
0	histone cluster 1, H2ag; histone cluster 1, H2ah; histone cluster 1, H2ai; histone cluster 1, H2ak; histone cluster 1, H2al; histone cluster 1, H2am		3988
3.27E-16	H2A histone family, member V		2521.9
1.03E-30	programmed cell death 11 (*Mem)*		1390.2
0`.000128	solute carrier family 30 (zinc transporter), member 7 (*Mem)*		1321.7
3.07E-12	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit *(Mem, CCR)*		1288.4
4.30E-105	cytochrome b5 type B (outer mitochondrial membrane) (*Mem)*		1221
1.30E-65	VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa (*Mem)*		1207.2
7.70E-162	H2A histone family, member X *(CCR, DNA damage repair)*		1194.2
2.30E-23	ribosomal protein L34 *(PS, Tr)*		1188.7
1.45E-15	hippocalcin like 4		1174.5
]1.40E-11	small nuclear ribonucleoprotein polypeptides B and B1		1121.7
0	tubulin, beta 4		1059.6
3.51E-42	ribosomal protein L23 pseudogene 6; ribosomal protein L23 *(PS, Tr)*		1025
7.80E-41	ribosomal protein S10; ribosomal protein S10 pseudogene 4; ribosomal protein S10 pseudogene 11 13 *(PS, Tr)*		1010.8
2.66E-71	chromosome 10 open reading frame 58; chromosome 10 open reading frame 57 (*Mem)*		1000.5
1.19E-17	ribosomal RNA processing 12 homolog (S. cerevisiae) *(PS, Tr, Mem)*		994.2
3.32E-39	heterogeneous nuclear ribonucleoprotein A0		991.6
1.84E-26	ribosomal protein L26 pseudogene 33; ribosomal protein L26; ribosomal protein L26 pseudogene 16; ribosomal protein L26 pseudogene 19; ribosomal protein L26 pseudogene 6 *(PS, Tr)*		987.7
7.87E-18	transmembrane emp24 domain trafficking protein 2 *(Mem)*		985
2.76E-12	asparaginyl-tRNA synthetase *(PS, CCR)*		970
3.30E-148	RAB14, member RAS oncogene family (*Mem)*		960.7
2.10E-120	microsomal glutathione S-transferase 1 (*ResOS, Mem)*		943.1
3.68E-08	U2 small nuclear RNA auxiliary factor 2		941.6
3.99E-17	ribosomal protein L18a pseudogene 6; ribosomal protein L18a *(PS, Tr)*		939.3
2.20E-17	C-type lectin domain family 16, member A		938.8
0	heterogeneous nuclear ribonucleoprotein A3		934.9
8.96E-10	RAN binding protein 2		882.1
1.82E-06	SEC11 homolog A (S. cerevisiae) (*Mem)*		868.4
1.33E-68	splicing factor, arginine/serine-rich 9		866.7
9.48E-05	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2, 14.5kDa (*Mem)*		856.2
2.19E-28	polyribonucleotide nucleotidyltransferase 1		825.3
1.89E-36	cytochrome c oxidase subunit IV isoform 1 (*Mem)*		819.3
7.43E-12	chromosome 7 open reading frame 50		819
1.40E-134	RAB2A, member RAS oncogene family (*Mem)*		803.6
1.39E-26	ADP-ribosylation factor 4		787.4
3.00E-117	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit B1 (*Mem, CCR)*		770
1.01E-06	RecQ protein-like (DNA helicase Q1-like) *(CCR, DNA damage repair)*		769.5

9.05E-62	ribosomal protein, large, P2 pseudogene 3; ribosomal protein, large, P2 *(PS, Tr)*	768.2
4.92E-11	ilvB (bacterial acetolactate synthase)-like *(Mem)*	729.5
3.53E-18	hepatoma-derived growth factor (high-mobility group protein 1-like)	729.5
1.88E-07	ADP-ribosylation factor-like 8B (*Mem*)	727.2
1.58E-14	ENSG00000174886	727.1
1.60E-148	phosphatidylethanolamine binding protein 1 (*ROS, Mem, CCR*)	700.6
1.07E-37	GCN1 general control of amino-acid synthesis 1-like 1 (yeast)	699
2.29E-42	cytochrome b5 type A (microsomal) (*Mem*)	675.4
0	serpin peptidase inhibitor, clade B (ovalbumin), member 12	674
7.73E-67	ribosomal protein S9; ribosomal protein S9 pseudogene 4 *(PS, Tr)*	669.8
1.85E-26	adipose differentiation-related protein (*ResOS, Mem*)	658.9
3.86E-55	ribosomal protein S6 pseudogene 25; ribosomal protein S6; ribosomal protein S6 pseudogene 1 *(PS, Tr, Apop)*	658.2
4.50E-64	S100 calcium binding protein P	654
0.000505	splicing factor 3b, subunit 4, 49kDa	639.9
1.37E-28	high-mobility group box 1; high-mobility group box 1-like 10 (*Apop, DNA damage repair)*	633.7
3.11E-40	thioredoxin-related transmembrane protein 2 *(Mem)*	624.8
1.42E-14	BCS1-like (yeast) (*Mem, CCR*)	621.3
1.80E-147	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*Tr, Apop*)	612.8
5.52E-41	chromosome 9 open reading frame 114	605.6
6.83E-06	scribbled homolog (Drosophila) *(Apop, Mem)*	600.9
2.16E-34	SERPINE1 mRNA binding protein 1	595
1.12E-11	RAN binding protein 6	590.1
9.66E-47	eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa (PS*, Tr, CCR*)	587.6
2.78E-46	echinoderm microtubule associated protein like 4 *(CCR)*	585.7
8.10E-283	signal recognition particle receptor, B subunit (*Mem*)	579.8
7.06E-06	laminin, alpha 5	560.7
2.60E-125	complement component 1, q subcomponent binding protein	559.1
7.58E-16	mitochondrial ribosomal protein L37	558.3
5.11E-08	methionyl-tRNA synthetase *(PS, CCR)*	557.7
1.12E-30	ENSG00000215472	554.1
5.54E-95	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 4; sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3	552.2
1.44E-25	gamma-glutamyl cyclotransferase *(Apop)*	546.5
6.08E-12	isocitrate dehydrogenase 3 (NAD+) alpha	542.8
2.80E-249	glyceraldehyde-3-phosphate dehydrogenase-like 6; hypothetical protein LOC100133042; glyceraldehyde-3-phosphate dehydrogenase *(Mem)*	542.1
8.76E-11	Fas associated factor family member 2	529.9
9.16E-30	tetratricopeptide repeat domain 35	514.6
8.36E-25	ribosomal protein S15a pseudogene 17; ribosomal protein S15a pseudogene 19; *(PS, Tr)*	507.8
2.01E-12	CTAGE family, member 5 pseudogene; CTAGE family member; CTAGE family, member 4; CTAGE family, member 5 *(Mem)*	506.4
7.40E-130	ribosomal protein L9; ribosomal protein L9 pseudogene 25 *(PS, Tr)*	497.5
1.00E-115	RAB1A, member RAS oncogene family (*Mem, CCR*)	495.9
1.92E-52	ribosomal protein S16 pseudogene 1; ribosomal protein S16 pseudogene 10; ribosomal protein S16 *(PS, Tr)*	494.7
0.000776	LIM homeobox transcription factor 1, beta	489.7
1.33E-82	VAMP (vesicle-associated membrane protein)-associated protein B and C *(ResOS, Mem)*	487.2

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1.76E-13	Mov10, Moloney leukemia virus 10, homolog (mouse) *(Mem)*	485.1
2.24E-55	ribosomal protein S13 pseudogene 8; ribosomal protein S13; ribosomal protein S13 pseudogene 2*(PS, Tr)*	483.6
1.33E-52	RAB11B, member RAS oncogene family (*Mem*)	478.2
1.25E-11	isovaleryl Coenzyme A dehydrogenase *(CCR)*	477.8
8.37E-20	CSE1 chromosome segregation 1-like (yeast) *(Apop)*	472.5
0.000761	proteasome (prosome, macropain) subunit, alpha type, 4 *(CCR)*	469.4
2.52E-17	ribosomal protein L15 pseudogene 22;	468.5
4.86E-11	eukaryotic translation initiation factor 2-alpha kinase 2 *(PS, ResOS, CCR, Apop)*	467.6
5.39E-06	SAR1 homolog B (S. cerevisiae) (*Mem*)	467.6
4.94E-27	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12 (*Mem*)	466.3
1.05E-08	ribosomal protein L27 (*PS, Tr*)	463.6
3.09E-40	glutamine-fructose-6-phosphate transaminase 1	462.2
1.05E-33	aspartyl-tRNA synthetase *(PS, CCR)*	461.3
3.05E-05	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa (*Mem*)	449.2
2.93E-10	zinc finger RNA binding protein	448.4
2.64E-55	proliferation-associated 2G4, 38kDa; proliferation-associated 2G4 pseudogene 4 *(CCR)*	445.6
7.65E-35	talin 1 *(Mem)*	444.4
2.88E-12	LEM domain containing 2 *(Mem)*	443.7
4.50E-240	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6 (*Mem, Apop*)	441.3
7.06E-05	chromobox homolog 1 (HP1 beta homolog Drosophila)	434.9
4.75E-30	Thioredoxin	433.9
1.80E-37	ribosomal protein L21 pseudogene 134; ribosomal protein L21 pseudogene 80; ribosomal protein protein L21 *(PS, Tr)*	430.8
1.48E-18	proteasome (prosome, macropain) subunit, beta type, 1 *(CCR)*	428.6
1.10E-10	inositol 1,4,5-triphosphate receptor, type 3 *(Mem)*	427.2
1.50E-178	ER lipid raft associated 1 *(Mem)*	427.1
1.89E-41	ribosomal protein L13a pseudogene 7; ribosomal protein L13a pseudogene 5; ribosomal protein L13a pseudogene 16; ribosomal protein L13a; ribosomal protein L13a pseudogene 18 *(PS, Tr)*	426.6
2.99E-22	v-ral simian leukemia viral oncogene homolog A (ras related) *(Mem)*	423.9
2.96E-08	RAN, member RAS oncogene family (*Tr, CCR*)	423.8
2.63E-14	retinol dehydrogenase 11 (all-trans/9-cis/11-cis) *(Mem)*	418.5
5.30E-122	eukaryotic translation initiation factor 3, subunit L*(PS)*	413.3
9.36E-44	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	412.5
4.13E-51	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa *(PS)*	411.8
7.20E-102	prolactin regulatory element binding *(Mem)*	411.8
1.82E-06	emopamil binding protein (sterol isomerase) (*Mem*)	411.6
1.56E-26	RAP1B, member of RAS oncogene family (*Mem*)	409.1
7.96E-07	NADH-ubiquinone oxidoreductase chain 5 (*Mem*)	408.9
1.32E-40	ribosomal protein S25 pseudogene 8; ribosomal protein S25 *(PS, Tr)*	407.5

	Gene Name **RET**	Percentage of control ion intensity
0	histone cluster 1, H2ag; histone cluster 1, H2ah; histone cluster 1, H2ai; histone cluster 1, H2ak; histone cluster 1, H2al; histone cluster 1, H2am	4813.246
1.19E-17	ribosomal RNA processing 12 homolog (S. cerevisiae) (Mem)	956.2
1.02E-12	ATP citrate lyase	824.9
1.03E-30	programmed cell death 11 (Apop)	692.1

PEP	Gene Name		Percentage of control ion intensity
1.34E-07	nucleolar complex associated 3 homolog (S. cerevisiae)		672.4
1.37E-28	high-mobility group box 1; high-mobility group box 1-like 10 (Apop, DNA damage repair)		650
2.34E-08	sel-1 suppressor of lin-12-like (C. elegans) (Mem)		647.3
3.09E-14	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (Mem)		600
7.06E-06	laminin, alpha 5		534.7
4.71E-17	catenin (cadherin-associated protein), delta 1 (Mem)		524.4
6.45E-43	pentatricopeptide repeat domain 1		455.6
5.69E-05	growth arrest and DNA-damage-inducible, gamma interacting protein 1 (PS, CCR)		418.6
1.82E-06	emopamil binding protein (sterol isomerase) (Mem)		417.8

PEP	Gene Name	**DON/RET**	Percentage of control ion intensity
1.07E-26	uridine phosphorylase 2		14920.8
2.75E-08	lectin, galactoside-binding, soluble, 7; lectin, galactoside-binding, soluble, 7B		1676.3
5.67E-58	KIAA0020		1412.3
0.012821	spermatogenesis associated 18 homolog (rat)		1393.9
6.83E-06	scribbled homolog (Drosophila)		682.2
4.75E-30	Thioredoxin		663.6
9.81E-53	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide		648.9
1.47E-43	enhancer of rudimentary homolog (Drosophila)		558.7
0	signal recognition particle 14kDa (homologous Alu RNA binding protein) pseudogene 1; signal recognition particle 14kDa (homologous Alu RNA binding protein)		515.4
5.74E-09	polymerase (RNA) I polypeptide C, 30kDa		515.1
3.09E-40	glutamine-fructose-6-phosphate transaminase 1		489.0
1.51E-65	glutathione reductase		478.0
0	ribosomal protein L9; ribosomal protein L9 pseudogene 25		450.5
7.80E-41	ribosomal protein S10; ribosomal protein S10 pseudogene 4; ribosomal protein S10 pseudogene 11; ribosomal protein S10 pseudogene 22; ribosomal protein S10 pseudogene 7; ribosomal protein S10 pseudogene 13		443.0
5.96E-23	chaperonin containing TCP1, subunit 5 (epsilon)		424.5
1.98E-43	chaperonin containing TCP1, subunit 7 (eta)		421.8
2.16E-06	COX4 neighbor		419.7
1.18E-08	proteasome (prosome, macropain) 26S subunit, ATPase, 1; similar to protease (prosome, macropain) 26S subunit, ATPase 1		413.0

Table 10.2. Reduced expression of protein groups compared with the control for HepG2 cells exposed to 10 µM DON, RET or both for 48 h. Each protein group is shown with the PEP value which is the significance probability of the identification ranging from <0.001 as a minimum to zero (absolute identification). Only a 4-fold reduction (25% of control or less) was considered significant and is presented. Functionality of each protein is grouped as previously and labelled as follows: PS (protein synthesis); Tr (translational elongation), Apop (apoptosis); Mem (membrane); ResOS (response to organic substance) and CCR (cell cycle regulation).

PEP	Gene name	**DON**	Percentage of control ion intensity

0	protein disulfide isomerase family A, member 6	24.9
2.49E-07	similar to ferritin, light polypeptide; ferritin, light polypeptide	24.7
3.19E-06	dihydrolipoamide branched chain transacylase E2	24.6
8.20E-144	translocase of outer mitochondrial membrane 7 homolog (yeast) (*Mem*)	24.5
1.35E-07	Ly1 antibody reactive homolog (mouse)	24.1
3.32E-27	alcohol dehydrogenase 4 (class II), pi polypeptide	23.9
7.96E-30	eukaryotic translation elongation factor 1 epsilon 1	23.8
1.40E-21	phosphate cytidylyltransferase 1, choline, alpha *(Mem)*	23.3
6.84E-10	acyl-Coenzyme A oxidase 2, branched chain	23.1
7.42E-07	dipeptidase 1 (renal) *(Mem)*	23
5.80E-45	acyl-Coenzyme A oxidase 1, palmitoyl	22.5
8.17E-05	serpin peptidase inhibitor, clade B (ovalbumin), member 1	22.3
3.06E-84	endoplasmic reticulum protein 29	22
0.000167	hypothetical LOC100130009; high mobility group AT-hook 1	21.4
1.18E-05	cathepsin B	21.3
8.10E-135	hydroxysteroid dehydrogenase like 2	21.3
4.61E-32	secretory carrier membrane protein 2 *(Mem)*	21.1
8.00E-208	vesicle amine transport protein 1 homolog (T. californica)	20.2
7.74E-05	N-myc downstream regulated 1 *(Mem)*	20.2
1.70E-14	transcription elongation factor A (SII), 3	20
5.99E-79	heat shock 27kDa protein-like 2 pseudogene; heat shock 27kDa protein 1	19.4
1.66E-06	myelin protein zero-like 1	19.1
2.80E-126	serine hydroxymethyltransferase 2 (mitochondrial)	18.9
3.74E-06	protein O-fucosyltransferase 1	18.6
3.19E-13	carboxypeptidase D *(Mem)*	18.6
2.01E-07	glycosyltransferase 25 domain containing 1	18.3
9.70E-138	hypothetical LOC100129500; apolipoprotein E	17.6
0	histone cluster 1, H1c	17.3
3.90E-138	annexin A7	17
5.30E-290	mercaptopyruvate sulfurtransferase	17
4.59E-15	catechol-O-methyltransferase *(Mem)*	16.8
6.82E-15	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8kDa (*Mem*)	16.5
9.75E-09	HtrA serine peptidase 2 *(Mem, Apop)*	15.6
9.64E-12	mitochondrial ribosomal protein L39	15.3
4.72E-07	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial)	14.6
3.20E-07	arylsulfatase E (chondrodysplasia punctata 1)	14
3.25E-12	FUN14 domain containing 2	13.6
3.34E-08	lectin, galactoside-binding, soluble, 3 binding protein	13.4
9.84E-05	sialidase 1 (lysosomal sialidase) *(Mem)*	12.8
6.12E-61	farnesyl-diphosphate farnesyltransferase 1 (*Mem*)	12.7
5.56E-38	PDZ and LIM domain 1	12.5
1.96E-27	tropomyosin 4	11.5
1.03E-07	transcription factor B1, mitochondrial	11.2
2.59E-26	poly(rC) binding protein 2	10.6
0.000853	translocase of outer mitochondrial membrane 6 homolog (yeast) (*Mem*)	9.6

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PEP	Gene Name	Percentage of control ion intensity
2.42E-13	carbonic anhydrase I	9.4
5.93E-07	cytochrome P450, family 4, subfamily F, polypeptide 2	9
2.57E-32	chromosome 11 open reading frame 59 *(Mem)*	8.8
1.78E-05	ribosomal protein S29 pseudogene 11; ribosomal protein S29 pseudogene 16; *(PS)*	8.6
2.49E-22	small nuclear ribonucleoprotein polypeptide E-like 1; small nuclear ribonucleoprotein polypeptide E; similar to hCG23490	8.1
1.77E-11	ribosomal protein L28	3.2

PEP	Gene Name **RET**	Percentage of control ion intensity
0.001759	ADAM metallopeptidase domain 10	24.9
1.78E-05	ribosomal protein S29 pseudogene 11;	24.7
2.51E-16	KRR1, small subunit (SSU) processome component, homolog (yeast)	24.2
3.01E-79	protein kinase C substrate 80K-H	23.6
3.03E-12	general transcription factor II, i; general transcription factor II, i, pseudogene	22.5
1.66E-06	myelin protein zero-like 1	21.8
2.68E-06	adenine phosphoribosyltransferase	21.7
3.68E-08	U2 small nuclear RNA auxiliary factor 2	20.9
0.00E+00	small nuclear ribonucleoprotein D3 polypeptide 18kDa	17.0
0.000197	peptide deformylase (mitochondrial); component of oligomeric golgi complex 8	16.0
7.96E-30	eukaryotic translation elongation factor 1 epsilon 1	15.5
0.002203	vacuolar protein sorting 8 homolog (S. cerevisiae)	14.6
5.43E-13	calmodulin-like 5	13.6
7.42E-07	dipeptidase 1 (renal) *(Mem)*	10.7
2.42E-13	carbonic anhydrase I	10.4
0.005129	programmed cell death 6 interacting protein	7.9
1.77E-11	ribosomal protein L28	5.3
0.021621	phospholipase C, eta 1	4.9
2.38E-17	proline rich 4 (lacrimal)	3.2
0	lactate dehydrogenase A	0.1

PEP	Gene Name **DON/RET**	Percentage of control ion intensity
1.85E-16	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	25.0
2.28E-21	mago-nashi homolog, proliferation-associated (Drosophila)	24.7
1.4E-42	mitochondrial ribosomal protein S27	24.5
3.01E-79	protein kinase C substrate 80K-H	24.4
3.07E-12	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	24.3
5.6E-18	solute carrier family 16, member 3 (monocarboxylic acid transporter 4)	23.9
5.59E-13	transmembrane protein 70	23.8
6.02E-52	pyruvate carboxylase	23.7
2.31E-06	solute carrier family 2 (facilitated glucose transporter), member 2	23.7
1.6E-158	catenin (cadherin-associated protein), beta 1, 88kDa	23.5
2.85E-09	S100 calcium binding protein A16	23.2
2.1E-13	tripartite motif-containing 28	22.7
7.27E-68	poly(A) binding protein, nuclear 1	22.5
2.32E-24	acylglycerol kinase	22.5
5.42E-43	fibrinogen alpha chain	22.4

1.13E-05	P450 (cytochrome) oxidoreductase	22.0
8.8E-290	non-metastatic cells 1, protein (NM23A) expressed in; NME1-NME2 readthrough transcript; non-metastatic cells 2, protein (NM23B) expressed in	21.8
1.94E-08	cadherin 1, type 1, E-cadherin (epithelial)	21.0
0.002974	calcium activated nucleotidase 1	20.5
0.00189	syndecan 2	19.7
2.91E-53	topoisomerase (DNA) I	19.7
2.68E-06	adenine phosphoribosyltransferase	18.9
6.5E-113	myosin, heavy chain 9, non-muscle	18.7
9.72E-22	protein kinase, interferon-inducible double stranded RNA dependent activator	18.6
2.43E-73	adenylate kinase 3	18.4
2.61E-48	AHNAK nucleoprotein	18.2
1.43E-43	HEAT repeat containing 1	18.2
0	histone cluster 1, H2bo	18.1
1.62E-27	peroxiredoxin 4	18.1
5.9E-110	sterol carrier protein 2	18.1
4.17E-82	IQ motif containing GTPase activating protein 2	18.0
8.78E-18	nucleophosmin/nucleoplasmin, 3	18.0
0	histone cluster 1, H2bd	17.9
0.002826	lysocardiolipin acyltransferase 1	17.7
0.000172	zinc finger CCCH-type containing 15	17.2
0.001759	ADAM metallopeptidase domain 10	17.1
0	heat shock protein 90kDa beta (Grp94), member 1	17.0
7.96E-07	NADH-ubiquinone oxidoreductase chain 5	16.9
1.94E-42	glycoprotein, synaptic 2	16.9
1.01E-07	squamous cell carcinoma antigen recognized by T cells	16.6
4.8E-155	myosin IB	16.2
6.34E-40	tropomyosin 3	16.1
3.03E-12	general transcription factor II, i; general transcription factor II, i, pseudogene	16.1
1.01E-33	KH domain containing, RNA binding, signal transduction associated 1	15.3
6.28E-73	staphylococcal nuclease and tudor domain containing 1	14.6
3.09E-25	Nicastrin	14.1
3.3E-112	UDP-glucose ceramide glucosyltransferase-like 1	13.8
3.97E-06	spectrin repeat containing, nuclear envelope 2	13.3
1.3E-103	methionine adenosyltransferase II, alpha	13.0
9.73E-13	RNA binding motif (RNP1, RRM) protein 3	12.9
0.000197	peptide deformylase (mitochondrial); component of oligomeric golgi complex 8	12.4
4.03E-39	inter-alpha (globulin) inhibitor H2	12.4
6.87E-20	high mobility group AT-hook 2	12.3
3.07E-14	coatomer protein complex, subunit gamma	12.2
1.7E-19	solute carrier family 2 (facilitated glucose transporter), member 3	12.0
9.36E-36	glucosidase, alpha; neutral AB	11.9
1E-122	ribosomal protein S4, Y-linked 1	11.5
6.61E-46	similar to Bcl-2-associated transcription factor 1 (Btf); BCL2-associated transcription factor 1	11.3
0	glycyl-tRNA synthetase	10.7
6.7E-283	heterogeneous nuclear ribonucleoprotein U-like 2	10.6

5E-104	thyroid hormone receptor associated protein 3	10.1
5.43E-13	calmodulin-like 5	9.2
0	histone cluster 2, H2be	8.9
1.02E-54	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	8.4
4.86E-08	major vault protein	8.1
5.2E-52	hypoxia up-regulated 1	7.7
2.36E-08	cytochrome P450, family 27, subfamily A, polypeptide 1	5.8
0	fibronectin 1	4.9
0.021621	phospholipase C, eta 1	4.6
6.1E-122	alanyl (membrane) aminopeptidase	2.7
2.38E-17	proline rich 4 (lacrimal)	2.0
6.5E-174	actin, beta-like 2	1.5
0	lactate dehydrogenase A	0.1

10.2 Publication: "Biotransformation of patulin to hydroascladiol by *Lactobacillus plantarum"*

10.2.1 Authorship details, research roles and acknowledgements

Sumaiya Hawar approached Jane Beal and I through a mutual contact requesting to undertake the final year of her Baghdad University registered PhD at the University of Plymouth. We wrote a proposal together which encompassed many aspects aligned to my own research, with assays developed and optimised during the early part of my PhD, but this time with a different MT (PAT). I steered the research towards a productive endpoint and initiated the publishing of the data as the corresponding author. The initial spore germination assay, the HPLC analysis and growth curve assay was undertaken together; the RT-PCR was undertaken by Sumaiya Hawar and Sahar Karieb; the cellular toxicology, DNA sequencing, mass spectrometry and fragment analysis to identification of ascladiol E, Z and hydroascladiol was undertaken by myself; the writing of the manuscript was undertaken by Rich Billington and myself predominantly, with lesser input from the other authors. The amount of time spent on this project not only warranted a joint first authorship, but also a mention in the current thesis, especially the work not included in the published article or appearing in Sumaiya Hawar's PhD thesis (*i.e.,* cellular toxicology, mass spectrometry, DNA sequencing).