Factors influencing mycotoxin production in grass silage, and their effects on rumen metabolism and the microbiome in dairy cows

A thesis submitted in partial fulfilment of the requirements of

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by

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I. Declaration

I wish to declare that all of the work presented in this thesis is that of my own, unless stated otherwise. No portion of the work referred to in this thesis has been submitted in support of an application for another degree nor qualification of this or any other university, or institution of learning.

J. M. Webster September 2024

II. Abstract

Mycotoxins are secondary metabolites that can contaminate agricultural crops and forages (Eskola et al., 2020; Pusztahelyi et al., 2015) and are mainly synthesised by *Fusarium, Aspergillus* and *Penicillium* fungi, with harmful effects on humans and animals (Hussein and Brasel, 2001; Zain, 2011). Maize silage has been a focus of mycotoxin research as a matter of animal and public health, due to the acute toxicity of aflatoxin B_1, and the potential carry-over of this metabolite into milk (Min et al., 2021). In Great Britain, however, a considerable proportion of grass silage is produced for feeding dairy cows (Franco et al., 2021), where its mycotoxin profile has been understudied, and little is known of the effect on the rumen microbiome and metabolism. Previous studies identified mycotoxins of often *Penicillium* associated origin are dominant in grass silages, with non European Union (EU) regulated mycotoxins such as mycophenolic acid (MPA), present (O'Brien, 2010; Schneweis et al., 2000; Tangni et al., 2013). Most non-regulated grass silage mycotoxins identified to date, can elicit antibacterial and antifungal effects and even immunosuppressive effects (i.e. MPA (Heischmann et al., 2017)), that may lead to rumen dysbiosis and poorer dairy cow performance (Fink-Gremmels, 2008). Moreover, no correlation has been found between the presence of visible mould in silages and level of mycotoxin contamination (Manni et al., 2022), which could mean cows are exposed, unbeknownst to the farmer.

This thesis details a range of studies with the following aims: 1) to identify grass silage mycotoxins in Great Britain, 2) to elucidate effects of silage fermentation and management on mycotoxin production, 3) to understand the effect of grass silage mycotoxins on rumen fermentation, and also, 4) on the rumen microbiome, and dairy cow performance.

The findings of this thesis support that grass silage mycotoxin synthesis is influenced by fermentation parameters such as dry matter content, lactic acid and acetic acid concentration as well as management such as minimising oxygen proliferation through the clamp at opening. British grass silages often contain non EU regulated mycotoxins such as penicillic and fusaric acid that demonstrate the ability to alter rumen volatile fatty acid production, when in combination with other mycotoxins, at levels observed naturally synthesised on farm. Relative abundance of species of *Succiniclasticum*, *Methanobrevibacter* and *Prevotella* were impacted by the inclusion of MPA in the diet of dairy cows, with the potential to impact rumen function and animal performance, if fed for a prolonged duration. It highlights the necessity for regular mycotoxin testing on farm and for the EU Food and Safety Authority to consider synergistic mycotoxin combinations when deciding on guidance values in order to minimise impacts on the health and performance of cows in the British dairy industry.

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Nannie, I wish I could tell you about it all, but I think you probably always knew.

IV. Published work

Conference Proceedings

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IX. List of abbreviations

Abbreviation	Detail
Ac	Acetate
ADF	Acid detergent fibre
ADP	Adenosine diphosphate
AFB_1	Aflatoxin B_1
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
But	Butyrate
CFU	Colony forming units
CIT	Citrinin
CP	Crude protein
CYC	Cyclopiazonic acid
DIM	Days in milk
DM	Dry matter
DON	Deoxynivalenol
EMP	Embden-Meyerhof-Parnas pathway
ENN	Enniatin
ESI	Electrospray ionisation
EU	European Union
EtOH	Ethanol
FAO	Food and Agriculture Organisation
FUM	Fumonisins
FUS	Fusaric acid
GC	Gas chromatography
HAU	Harper Adams University
He LAB	Heterofermentative lactic acid bacteria
Ho LAB	Homofermentative lactic acid bacteria
HPLC	High performance liquid chromatography
IFM	In-vitro fermentation
JECFA	Joint Expert Committee on Food Additives
LAB	Lactic acid bacteria
LC/MS	Liquid chromatography/mass spectrometry
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LPD	Liquid phase digesta

MEA	Malt extract agar
MON	Moniliformin
MPA	Mycophenolic acid
MRS	De Man, Rogosa and Sharpe agar
ND	Not detected
NDF	Neutral detergent fibre
NIRS	Near-infrared spectroscopy
NR	Not reached
NS	Not significant
OTA	Ochratoxin A
OTB	Ochratoxin B
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PEN	Penicillic acid
Pi	Inorganic phosphate
PK	Phosphoketolase pathway
Prop	Propionate
RNA	Ribose nucleic acid
rRNA	Ribosomal RNA
s.e.d.	Standard error of the difference between means
SCC	Somatic cell count
TMR	Total mixed ration
UN	United Nations
UV	Ultra-violet
VFA	Volatile fatty acids
VRBGA	Violet-red bile glucose agar
WHO	World Health Organisation
ZEA	Zearalenone

Chapter 1

General introduction

1. General introduction

1.1. An introduction to silage

Ensiling is the process by which a feed or forage is preserved through acidification, via organic acids synthesised during microbial fermentation (Pahlow et al., 2015). This process of fermentation can therefore also increase the nutritive value of a feed (Kim et al., 2021). The fermented product, silage, has enabled farmers to provide sufficient nutrition to ruminant livestock all year round, particularly in temperate climates where grazing may be sub-optimal during winter months (Wilman et al., 2002). Instrumental in improving modern dairy cow performance through enhanced nutrition, silage is still a topic of great interest to farmers, scientists, and the feed industry (Koenig et al., 2023; Okoye et al., 2023).

1.1.1. A brief history of silage

Historically, humans have harnessed the power of bacterial and fungal fermentation pathways to preserve and produce food items (Hendy et al., 2021). There exists evidence of beer brewing, bread-making, and vinegar production as early as ancient Egypt, despite the lack of understanding at the time of the organisms involved (El-Mansi, 2018). The drying of agricultural crop such as grains, for storage was also practiced (Carrier, 1920). Anthropologists have speculated that human manipulation of fermentation processes long predates that of ancient Egypt, perhaps even carried out by early hominids to increase digestibility of certain roots or tubers (Hendy et al., 2021).

From the Egyptians, up until the 18th Century there exists almost no reference to the preservation of wet forage (Carrier, 1920). It was not until the 1700's where Italian farmers were observed preserving tree leaves in pits and about a hundred years later, when French farmers were also noted to be preserving fresh vine leaves for use in the production of Mount Dore cheese (Carrier, 1920) that the first example of the preservation of wet plant matter, was recorded. With regards to modern forage preservation for livestock feed however, it has been suggested that the earliest example most similar to the process that we know of as ensiling today, was practiced in 1850's Germany and Hungary, and included the salting of green fresh forage, compacted into pits and buried to form a sour fodder. The practice has been suggested to have possibly drawn influence from the production of "*sauerkraut*" (Carrier, 1920; Wilkinson et al., 2015).

1.1.2. The production of silage in the United Kingdom

The sour fodder production in Europe was investigated by British scientists in the 1800's, though hay-making remained the common method for the preservation of forage for livestock,

amongst British farmers. By this time, the preservation of maize by ensiling, had already spread to France and across to the United States, though popularity fluctuated amongst farmers in the United Kingdom (Brassley, 1996), perhaps due to the relative ease of pasture maintenance over the production of forage maize in the UK. It was not until 1950's Britain that a steady but consistent increase in the tons of grass and maize silage produced were observed over the decade (Figure 1-1). The promotion of ensiling fodder for cattle, by the Ministry of Agriculture, coupled with the end of the second World War may have relieved pressure on food security, allowing for more time and less risk aversion toward "experimental" farming (Brassley, 1996).

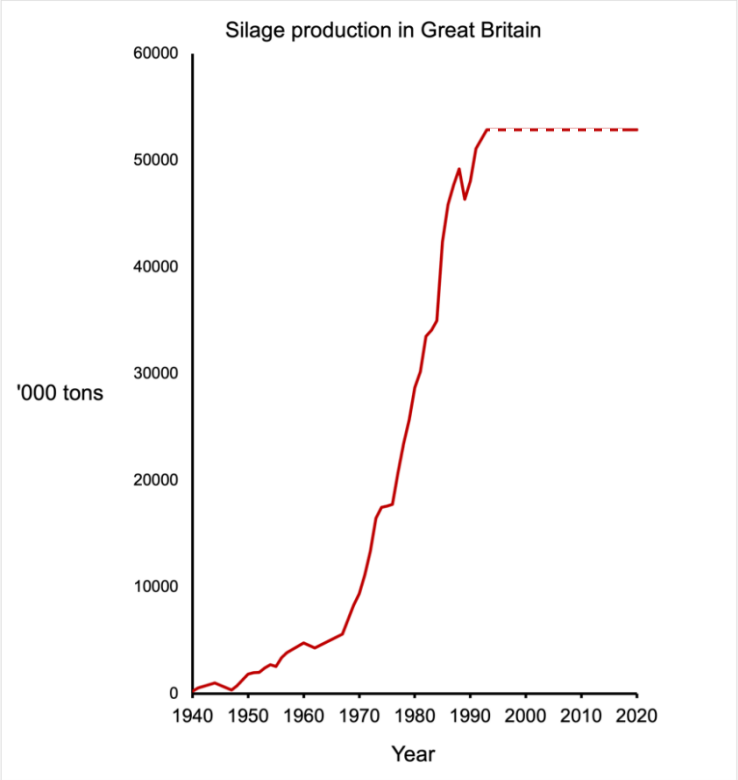


Figure 1-1. Silage production in 100,000 tons from the period of 1940 to modern day. Adapted from Brassley (1996) with Davies (2018). The dotted line indicates the period of time where data for silage production was not recorded.

Following the later decades of the 20th Century, the ease of grass silage production increased alongside the advent of improved agricultural technology (Wilman et al., 2002). In 1971, there were 11,130,000 tons of silage produced compared with the 540,000 tons of fresh weight produced in 1941 (Brassley, 1996). The recognition of the added nutritional quality that silage benefitted from over hay aided in the sharp uptake in the practice across the United Kingdom from the 1950's onwards (Wilkinson et al., 2015; Wilman et al., 2002). Silage production also

required less dependency on dry, clear weather than hay-making and most importantly, silage allowed farmers to hold more control over their herd's nutrition in contrast to traditional grazing systems (McDonald et al., 1991).

1.1.3. Silage today

Today, an estimated fifty million tonnes of fresh weight silage are produced every year in the United Kingdom alone (Davies, 2018), with grass silage the main forage used in winter feeding of cattle in the United Kingdom, Sweden, The Netherlands, Germany and the Republic of Ireland, to name a few (Van den Pol-van Dasselaar et al., 2019). It is also commonplace in the United Kingdom as well as in the United States for more intensive dairy systems to feed grass and maize silage as a large proportion of a complete diet throughout the whole year (Schingoethe, 2017). The adoption of a total mixed ration (TMR) system comprised of forages, grains and concentrates, has allowed farmers to benefit from continual monitoring of cow dietary intake, increasing profits through improved animal performance (Colman et al., 2011). Additionally, a TMR can be formulated specifically to provide targeted nutritional supplementation dependent on age and lactation stage, which is simply impractical in pure grazing systems (Schingoethe, 2017). Alongside improved selective breeding of dairy cows, underpinned by advanced genetic technology (Miglior et al., 2017), nutritional control has allowed for a more efficient conversion of feed into milk, and a reduction in yield losses due to ill health or a poor quality diet (Eastridge, 2006; Erickson and Kalscheur, 2019).

Genetic improvements to the modern dairy cow have allowed for greater milk yields, however, the metabolic pressure the modern cow faces has subsequently greatly increased also (Hansen, 2000; Oltenacu and Broom, 2010). The importance of producing a silage for a TMR with the greatest nutritional quality for the milking group is therefore vital, as even short-term dietary changes may lead to metabolic illness in the cow due to the demand of milk production on their physiology. Silage composition can therefore directly impact on the performance, efficiency and economics of high intensity dairy systems in the United Kingdom (Colman et al., 2011; Penagos-Tabares et al., 2023; Roche, 2006).

Despite the ability to improve animal performance, there has been increasing concern regarding the ratio of forage to concentrates in TMRs for intensive dairy systems. Diets that are rich in concentrates, or grains can negatively impact on rumen metabolism due to the higher availability of rapidly fermentable starches and a lower fibre content in high concentrate TMR's (Kesler and Spahr, 1964). In some cases, the rumen microbiome can be impacted severely, leading to conditions such as sub-acute ruminal acidosis (Ma et al., 2022; Plaizier et al., 2022). With the environmental and public pressure in the United Kingdom to improve

sustainability in the agricultural industry, the use of grasses in dairy diets will likely only increase (https://farming.campaign.gov.uk/; Hennessy et al., 2020; Koenig et al., 2023). Grass pastures themselves that are insufficient for the production of human feed, can often in part, be grazed by cattle, or used to produce silages; harnessing human inaccessible plant protein for the production of animal protein for human consumption (Hennessy et al., 2020). In grass silages, manipulation of the fermentation can allow for reductions in the use of supplementary concentrates in a TMR, and a reduction in production costs for the farmer (Ho et al., 2018; Rupp et al., 2021)

Therefore, it is unsurprising that there is such scientific and commercial interest in further understanding the microbiology of grass silage fermentation (Okoye et al., 2023; Rinne et al., 2022). Points of research focus have included management of the crop in-field, directed fermentation of the silage, inhibition of spoilage organisms and mitigation of loss to waste. The next sections will detail the process of ensiling, including the microbiology and the current "best practice" recommendations for the production of good quality grass silage for high intensity dairy systems in the United Kingdom.

1.1.4. The process of ensiling

The process of ensiling involves four main phases that can at any point influence the final nutritional quality of the silage and any wastage (Bolsen et al., 1996). In order, these are labelled the aerobic phase, fermentation phase, stable/storage phase, and the feed-out phase (McDonald et al., 1991; Pahlow et al., 2015). Though occurring prior to ensiling, factors at the point of harvest play an important role in the success of forage preservation so a fifth additional "harvest" phase, will be incorporated from here onwards (Borreani et al., 2018). During these five phases there are a multitude of interactions that continually take place between plant, bacteria and fungi. Simply, silage is formed when organic acids produced by epiphytic lactic acid bacteria acidify the silage environment, preserving it until it is again re-exposed to air on feeding-out (Pahlow et al., 2015).

For grass silage typically, the crop is mown, wilted, and chopped up whilst harvested from the field. At this point a silage additive (e.g. acids) or inoculant (e.g. bacteria) may be incorporated to aid in the acidification of the crop (Muck et al., 2018; Soundharrajan et al., 2021). The forage is then emptied in layers into a large pit or clamp and worked by machinery to compact the layers. Using tyres, gravel bags or bales, often a polyethylene sheet is weighed down over the forage to form an airtight seal (Wilkinson and Davies, 2013). Recommended "best practices" for making silage of a good nutritional quality, were reviewed in detail by Dunière et al., (2013) and include the following points:

1) Limitation of pathogen introduction at harvest.
2) Promoting establishment of anaerobiosis.
3) Promoting acidification.
4) Limiting air ingress during storage.
5) Improving aerobic stability.
6) Direct inhibition of undesirable organisms.

The applied management methods supporting these points above, with their respective phase of the process are detailed in Table 1-1.

Table 1-1. “Best practice” recommendations for production of good quality silage as described in the literature, including the relevant applied management practice.

Recommendations	Phase	Achieved by:	Reference
Limitation of pathogen introduction at harvest	Harvest	Minimisation of soil contamination at mowing, tedding, and harvesting.	Pahlow et al., (2015)
	Harvest	Reduction in unnecessary time spent wilting the crop. The longer the crop is left out to wilt, the higher the likelihood of the proliferation of spoilage organisms.	Hodulíková et al., (2016)
	Harvest	Ensure slurry/manure application has been given adequate time to be absorbed by the soil and the crop, before mowing and harvest.	Davies et al., (1996); Johansson et al., (2005)
Establishment and maintenance of an anaerobic environment and improve aerobic stability	Aerobic	Ensure forage is properly compacted to prevent air ingress.	McGechan and Williams, (1994); Snelling et al., (2023)
	All phases	Use an oxygen barrier sheet, or plastic sheets covering and to the sides of the silage.	McGechan and Williams (1994); Orosz et al., (2013)
	Feed-out	When cutting into the silage ensure no tearing occurs to reduce ingress of oxygen into the silage clamp. Utilisation of a shear-grab rather than bucket, will achieve this.	Wilkinson and Davies, (2013)
	Feed-out	Maintain plastic sheet coverings the clamp at feed-out.	Wilkinson and Davies (2013)

Table 1-1. (Continued)

Recommendations	Phase	Achieved by:	Reference
Ensuring a rapid acidification of the silage environment	All phases	Consideration of how the dry matter content and protein content of the forage may affect the acidification rate when ensiled, due to buffering effects of these factors.	Playne and McDonald, (1966); Wilson, (1935)
	Harvest	Addition of silage additives such as directly adding inorganic or organic acidic compounds to the forage.	Queiroz et al., (2018)
	Harvest	Addition of silage inoculants to increase the population of viable lactic acid bacteria present.	Gonda et al., (2023); Oliveira et al., (2017)
Inhibition of undesirable organisms	Fermentation Feed-out	Expected that a rapid acidification and maintenance of anaerobicity will prevent the growth of undesirable microorganisms during the fermentation such as *Enterobacteriaceae, Clostridia,* yeasts and filamentous fungi. However, despite achieving a successful fermentation, appreciate that some spoilage organisms or spores can survive the acidic conditions of the fermentation, and may proliferate later upon feed-out.	Dunière et al., (2013)
	Feed-out	Consider how the forage fermentation profile may impact on the aerobic stability of the silage and take precautions to mitigate aerobic spoilage, such as considering an appropriate feed-out rate.	Wilkinson, (2015)
	Feed-out	Monitor temperature of the clamp face at feed-out to assess extent of heating which indicates aerobic spoilage.	Le Cocq et al., (2020)

1.1.5. The microbiology of silage fermentation

There are a series of interactions that occur throughout the five phases of silage production between the forage and the epiphytic microbiota. Environmental factors and forage management can all influence certain aspects of the many microbiological processes that occur and are detailed in Figure 1-2.

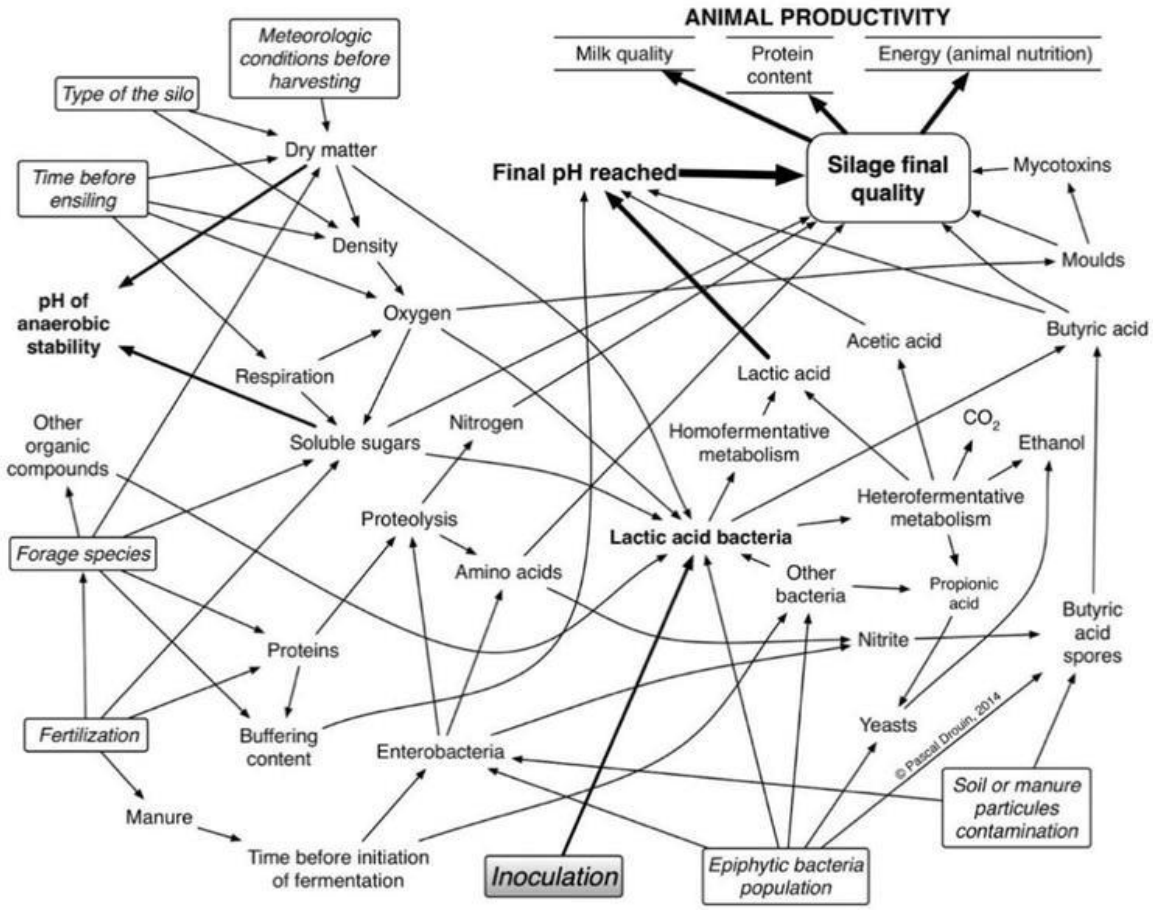


Figure 1-2. Overview of the microbial, environmental and forage management practices that influence silage preservation and the resultant animal performance. Figure from Drouin et al., (2020).

1.1.6. The lactic acid bacteria

Establishing an efficient fermentation first and foremost requires a viable population of lactic acid producing bacteria (Kim et al., 2021). Found naturally occurring on the crop, these epiphytic populations can contain species from the genera *Lactiplantibacillus, Lentilactobacillus, Lacticaseibacillus, Weissella* (formerly *Lactobacillus* and *Leuconostocaceae* (Zheng et al., 2020)), *Pediococcus, Enterococcus, Lactococcus* and

Streptococcus (Ávila and Carvalho, 2020; Pahlow et al., 2015). The Lactic acid bacteria (LAB) are best classified into two groups dependent on their carbohydrate fermentation pathway utilised; obligate homofermentative LAB and facultative heterofermentative LAB, and obligate heterofermentative LAB (Figure 1-3).

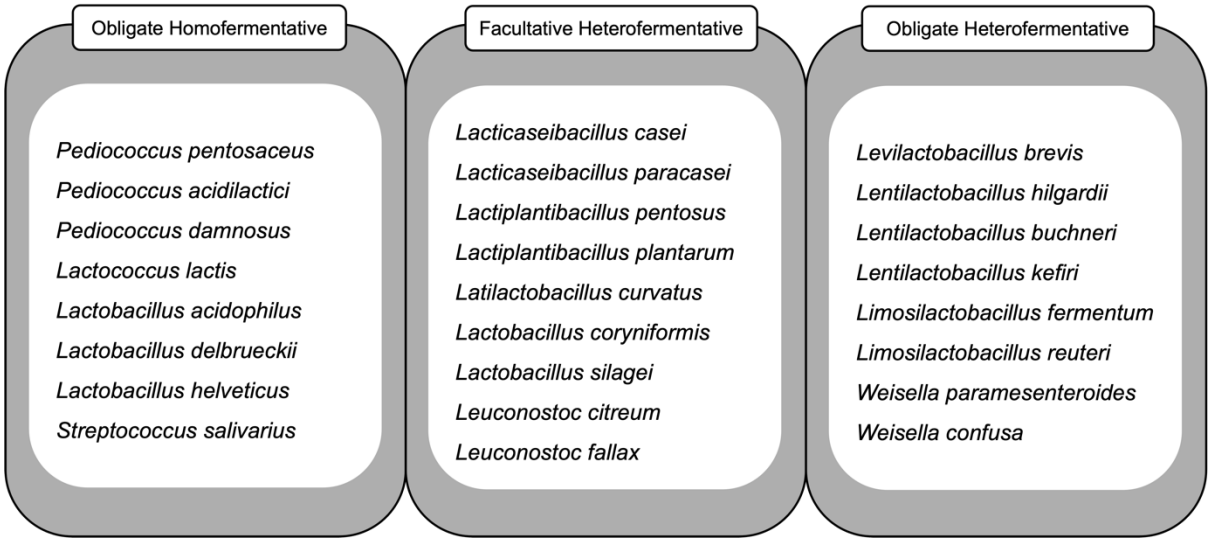


Figure 1-3. Classification of some genera and species of lactic acid bacteria as either obligately homofermentative, facultatively heterofermentative, or obligately heterofermentative. Adapted from Buron-Moles et al., (2019) with taxonomic updates according to Zheng et al., (2020).

1.1.6.1. Homofermentative lactic acid bacteria

Obligately homofermentative lactic acid bacteria such as *Pediococcus pentosaceus* utilise the Embden-Meyerhof-Parnas (EMP) pathway to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP) using the energy from the catabolism of hexose sugars (Eiteman and Ramalingam, 2015). Using the enzyme fructose bisphosphate, one mole of hexose sugar (i.e. glucose), can yield 2 moles of lactic acid (Equation 1). The EMP pathway is therefore favoured for the most efficient and rapid production of lactic acid in the silage environment with the most conservative use of carbohydrates from the grass. Obligate homofermentative LAB are unable to ferment pentose sugars as they lack the enzyme phosphoketolase (McDonald et al., 1991).

Equation 1. Hexose fermentation pathway of homofermentative and facultative heterofermentative bacteria (Embden-Meyerhof-Parnas pathway; McDonald et al., (1991)).

$$\text{Hexose} + 2\,\text{ADP} + 2\,\text{Pi} \rightarrow 2\,\text{Lactate} + 2\,\text{ATP} + 2\,\text{H}_2\text{O}$$

1.1.6.2. Heterofermentative lactic acid bacteria

Facultative heterofermentative LAB are also able to ferment hexose through the EMP pathway. Obligate heterofermentative LAB are able to utilise the phosphoketolase (PK; Equation 2) pathway only, exclusively yielding one mole each of lactate and acetate (Eiteman and Ramalingam, 2015) and in some cases mannitol from hexose sugars. Though still capable of lowering the pH of the silage environment, acetate has a comparatively higher pK_a (pK_a: 4.76) value than that of lactic acid (pK_a: 3.86; www.ncbi.nlm.nih.gov, 2024). The pK_a is calculated as the -log of the K_a, where the K_a is the dissociation constant of an acid, therefore the stronger an acid, the lower the pK_a value. High concentrations of acetate in silages have also been shown to impair the palatability and intake of the silage by livestock (Gerlach et al., 2021). Therefore the obligate heterofermentative LAB are less favourable in an efficient silage acidification compared to the facultative heterofermentative and obligate homofermentative LAB. Obligate heterofermentative LAB are also able to ferment pentose sugars (Equation 3) to form acetate and lactate, though the majority of the readily fermentable carbohydrates of grass, are in hexose form (McDonald et al., 1991).

Equation 2. Hexose fermentation pathways of obligate heterofermentative lactic acid bacteria (Phosphoketolase pathway; McDonald et al., (1991)).

$$\text{Hexose} + 2\,\text{ADP} + 2\,\text{Pi} \rightarrow \text{Lactate} + \text{Acetate} + 2\,\text{ATP} + 2\,H_2O$$

$$\text{Glucose} + 2\,\text{Fructose} + H_2O + 2\,\text{ADP} + 2\,\text{Pi} \rightarrow \text{Lactate} + \text{Acetate} + 2\,\text{Mannitol} + CO_2 + 2\,\text{ATP}$$

Equation 3. Pentose fermentation pathway of obligate heterofermentative lactic acid bacteria (Phosphoketolase pathway).

$$\text{Pentose} + 2\,\text{ADP} + 2\,\text{Pi} \rightarrow \text{Lactate} + \text{Acetate} + 2\,\text{ATP} + 2\,H_2O$$

Obligate heterofermentative LAB such as *Lentilactobacillus buchneri* are also able to ferment the lactic acid produced, into propionate and propane-1,2-diol, which is subsequently fermented to propan-1-ol and another mole of propionate (Equation 4; (Oude Elferink et al., 2001)). The pK_a of propionic acid (pK_a: 4.88) again is comparatively higher than acetic and lactic acids, resulting in a dilution of the acidity of the immediate silage environment, and a loss of fermentable carbohydrates to CO_2 gas. The inclusion of propane-1,2-diol (*propylene glycol*) in the diet of dairy cows has been demonstrated to mitigate ketosis in dairy cows

(Nielsen and Ingvartsen, 2004), and to potentially lower rumen methane production (Wang et al., 2021), though this requires further study. There is, however, a cost to the silage quality through producing propane-1,2-diol as this fermentation pathway leads to a greater loss of structural carbohydrates from the silage (i.e. a loss of dry matter (DM)) which may have been utilised by the rumen microbial community upon consumption of the silage.

Equation 4. Secondary fermentation pathways of obligate heterofermentative lactic acid bacteria (namely: *L. buchneri, L. parabuchneri* and *L. dioloverans*; Oude Elferink et al., (2001)).

$$2\,\text{Lactic acid} + \text{ADP} + \text{Pi} \rightarrow \text{Acetic acid} + \text{Propane} - 1,2 - \text{diol} + \text{CO}_2 + \text{ATP}$$

$$2\,\text{Propane} - 1,2 - \text{diol} + \text{ADP} + \text{Pi} \rightarrow \text{Propionate} + \text{Propan} - 1 - \text{ol} + \text{H}_2\text{O} + \text{ATP}$$

Some lactic acid bacteria are able to produce bacteriocins and anti-fungal compounds that have been demonstrated as effective against spoilage organisms in grass silage (Elyass et al., 2017; Wang et al., 2012). However, a sufficient enough production of these compounds by the epiphytic strains present in grass silages to inhibit all spoilage organism proliferation cannot be guaranteed. Primarily, reducing the pH of silage rapidly and effectively remains essential to preventing the growth of spoilage organisms and inhibiting secondary fermentations that can occur as a result of other bacterial species such as those of *Clostridia* and *Enterobacteriaceae* (Borreani et al., 2018).

1.1.7. Organisms associated with loss of grass silage DM

Organisms that result in losses of silage DM are predominantly bacterial species such as *Enterobacteriaceae* and *Clostridia* groups (Kung et al., 2018), filamentous fungi (*moulds*), and yeasts. Not only do these organisms decrease silage quality through the loss of availability of fermentable carbohydrates for lactic acid production, but they can also produce toxic secondary metabolites that can pose a risk to livestock upon exposure (Hussein and Brasel, 2001).

1.1.7.1. Bacterial spoilage organisms

Bacterial species of the genera *Clostridia* are epiphytic to the soil and compete against LAB for the available plant fermentable carbohydrates (Jonsson, 1991). *Clostridia* produce butyric acid from hexoses, with a further loss of organic matter through the production of 2 moles of CO_2 gas per every mole of hexose sugar (Equation 5; McDonald et al., (1973)). A secondary fermentation pathway can also be carried out by *Clostridia*, depleting the lactate produced in the silage environment, yielding butyrate and 2 moles of CO_2 again. Silages high in butyric

acid content have been demonstrated to lead to hyperketonaemia in dairy cows (Andersson and Lundström, 1985; Vicente et al., 2014).

Equation 5. Hexose and lactate fermentation pathways carried out by *Clostridia,* McDonald et al., (1973).

$$\text{Hexose} + 3\,\text{ADP} + 3\,\text{Pi} \rightarrow \text{Butyrate} + 2\,\text{CO}_2 + 2\,\text{H}_2 + 3\,\text{ATP} + 3\,\text{H}_2\text{O}$$

$$2\,\text{Lactate} + \text{ADP} + \text{Pi} \rightarrow \text{Butyrate} + 2\,\text{CO}_2 + 2\,\text{H}_2 + \text{ATP} + \text{H}_2\text{O}$$

Toxins can also be produced by *Clostridia,* for example the species *C. botulinum*, which is responsible for botulism in livestock (Lindström et al., 2010), though this is uncommon in silage that has been contaminated by *Clostridial* species (Pahlow et al., 2015). The growth of *Clostridia,* however, can encourage the proliferation of toxin producing fungi in the silage environment due to the increase in pH, following the reduction in lactic acid concentration (Borreani et al., 2018). Increasing the pH can also allow other unfavourable organisms to re-establish their population, whom had previously been inhibited by the acidity. In wetter grass silages of a DM content of less than 300 g/kg, or in cases of heavy soil contamination upon ensiling, the risk of *Clostridia* contamination is greatly increased (Ávila and Carvalho, 2020; Haigh, 1990).

Another bacterial group responsible for the loss of organic matter in grass silages would be the genus *Enterobacteriaceae*. Species of bacteria of this genus, also compete with LAB for the hexose sugars available, yielding one mole of acetate and one mole of ethanol, as well as 2 moles of CO_2 gas. Similar to the less desirable fermentation carried out by obligate heterofermentative bacteria, the production of acetate instead of lactate, reduces the efficiency of the decline in pH due to the higher pK_a of acetate. There is a loss of organic matter to the production of carbon dioxide and the production of ethanol. A study by Yuan et al., (2016) suggested that the inclusion of ethanol in silage alongside an additive of *L. plantarum* improved the aerobic stability of grass silage compared to the control. Perhaps due to the bactericidal properties of ethanol (Sauerbrei, 2020) inhibiting the growth of spoilage bacteria upon exposure of silage to the air. However, a silage high in ethanol content may have consequences on the rumen microbial activity, and animal performance (Emery et al., 1959).

Equation 6. Hexose fermentation pathway of *Enterobacteriaceae,* Rooke and Hatfield, (2003).

$$\text{Hexose} + 3\,\text{ADP} + 3\,\text{Pi} \rightarrow \text{Acetate} + \text{Ethanol} + 2\,CO_2 + H_2O + 3\,\text{ATP}$$

1.1.7.2. Fungal spoilage organisms

Yeasts also compete with LAB for readily fermentable carbohydrates, forming ethanol and CO_2 which as previously stated are undesirable fermentation end-products (Equation 7).

Equation 7. Hexose fermentation pathway of yeasts, McDonald et al., (1973).

$$\text{Hexose} + 2\,\text{ADP} + 2\,\text{Pi} \rightarrow 2\,\text{Ethanol} + 2\,CO_2 + 2\,\text{ATP} + 2\,H_2O$$

Filamentous fungi are also part of the epiphytic microbiota of the grass ensiled and are capable of fermenting carbohydrates through the EMP pathway. A series of fungi of the genera *Aspergillus*, *Claviceps, Fusarium,* and *Penicillium* can contaminate grasses and are able to produce secondary metabolites with toxic effects on the livestock exposed to them (Buszewska-Forajta, 2020; Wambacq et al., 2016). Filamentous fungi are less easy to inhibit in silage as some fungal species can lay dormant in spore-form (Ajmal et al., 2022) or remain unaffected by the acidity achieved during ensiling due to the ability of fungi to detect pH and modulate their immediate environment (Vylkova, 2017). Fungal metabolites, referred to as mycotoxins, are explored further in section 1.2.

1.1.8. Additives and inoculants

Reducing silage losses of organic matter to spoilage organisms can be mitigated by encouraging a lactic acid fermentation (Borreani et al., 2018; Davies et al., 2018; Wróbel et al., 2023). This can be promoted by the addition of chemical additives, bacterial inoculants or enzymes, that can be added during clamp filling or during harvest to directly impact on the initial fermentation or improve silage aerobic stability (Muck et al., 2018; Puntillo et al., 2020). Silage additives are designed to be “inhibitory” against spoilage organisms and include the direct application of compounds such as formic, acetic and hydrochloric acids, formaldehyde, or sodium hydroxide to the forage before ensiling (Muck, 2010). This practice has decreased in popularity however, in part due to the greater ease and safety of application of LAB inoculants relative to these inhibitory compounds (Okoye et al., 2023). Silage LAB inoculants are largely comprised of “generally recognised as safe” (GRAS) bacteria (Mejía-Avellaneda et al., 2022) and produce additional organic acids for rumen metabolism (Kim et al., 2021). Studies have also reported beneficial bacteriocins that can be produced by certain LAB strains

(Elyass et al., 2017), antifungals (Lee et al., 2021), and some isolated strains have demonstrated an ability to degrade certain mycotoxins (Fabiszewska et al., 2019; Niderkorn et al., 2006).

Often an inoculant bacterial combination is tailored with the target of increasing the starting populations of certain lactic acid bacteria favouring either homolactic or heterolactic fermentations or a combination of both (Drouin et al., 2020). A range of inoculants currently available commercially in the United Kingdom, their bacterial composition and their intended fermentation direction, are listed in Table 1-2.

Table 1-2. A selection of grass silage inoculants commercially available in the UK (product names and formulations are correct as of August 2024). Not all available products are listed.

Inoculant name	Manufacturer name	Bacterial composition	Fermentation direction
Egalis® Ferment	Alltech®	*Lactiplantibacillus plantarum* *Pediococcus pentosaceus*	Homo
BioStabil® Plus	Biomin® (DSM®)	*Lactiplantibacillus plantarum* *Levilactobacillus brevis* *Lentilactobacillus kefiri*	Combined
PIONEER 1188	Corteva®	*Lactiplantibacillus plantarum* *Enterococcus faecium*	Homo
OptiSile® Extra	EnviroSystems	*Lactiplantibacillus plantarum* DSM 19437 *Levilactobacillus brevis* DSM 23231 *Lentilactobacillus kefiri* DSM 19455	Combined
Powerstart®	Genus (ABS®)	*Lactiplantibacillus plantarum* AberF1	Homo

Table 1-2. (continued)

Inoculant name	Manufacturer name	Bacterial composition	Fermentation direction
Activator Plus	Kelvin Cave Ltd.	*Lactiplantibacillus plantarum* *Pediococcus pentosaceus*	Homo
LALSIL® Dry	Lallemand	*Pediococcus acidilactici* MA 18/5 U *Lentilactobacillus buchneri* NCIMB 40788	Combined
MAGNIVA® Platinum (2 & 3)	Lallemand	*Lentilactobacillus buchneri* NCIMB 40788 *Lentilactobacillus hilgardii* CNCM I-4785	Hetero
Sure-Sile® Fructan	Microferm	*Lactiplantibacillus plantarum* DSMZ 16627 *Pediococcus acidilactici* NCIMB 30005 *Lacticaseibacillus paracasei* NCIMB 30151	Homo
BONSILAGE® Forte	Schaumann	*Pediococcus acidilactici* *Lacticaseibacillus paracasei* *Lactococcus lactis*	Homo
BONSILAGE® Plus	Schaumann	*Lactiplantibacillus plantarum* *Pediococcus pentosaceus* *Lacticaseibacillus rhamnosus* *Levilactobacillus brevis* *Lentilactobacillus buchneri*	Combined
F1 ICE GOLD	Trevor Birchall Agriculture	*Lactiplantibacillus plantarum* DSMZ 15627 *Lacticaseibacillus paracasei* NCIMB30151 *Pediococcus acidilactici* NCIMB 30005 *Pediococcus pentosaceus* DSM 322291	Homo
Advance®	Volac®	*Pediococcus pentosaceus* *Lactiplantibacillus plantarum* *Levilactobacillus brevis*	Combined

Table 1-2 (continued)

Inoculant name	Manufacturer name	Bacterial composition	Fermentation direction
Ecocool®	Volac®	*Lactiplantibacillus plantarum* MTD/1 *Lentilactobacillus buchneri* PJB/1	Combined
Ecosyl® 100	Volac®	*Lactiplantibacillus plantarum* MTD/1	Homo

Genus names have been updated according to (Zheng et al., 2020) from the listed bacteria as per the manufacturer's information. Details on strain have been provided if known. Combined = combined homofermentative and heterofermentative fermentations, Homo = Homofermentative fermentation, Hetero = Heterofermentative fermentation.

Despite the ability of lactic acid bacteria to effectively inhibit the growth of *Clostridia* and *Enterobacteriaceae* there is little information on the relationship between lactic acid bacteria and filamentous fungi. Evidence suggests that some species of lactic acid bacteria, may be able to degrade certain mycotoxins in forages, but not all LAB strains are effective against all mycotoxins, and they may not completely eliminate all mycotoxins present in a silage (Antonio Gallo et al., 2021). Sporulating and filamentous fungi are able to tolerate the acidic conditions yielded by LAB (Park et al., 1996), often in a state of dormancy, or by altering their immediate environment (Vylkova, 2017).

It has been demonstrated that silage that has undergone a poor fermentation and experienced aerobic spoilage typically presents visible mould (O'Brien, 2010), but there is no correlation between extent or type of visible mould and concentration or profile of mycotoxins present (Manni et al., 2022). Indeed, silages that have undergone a seemingly successful fermentation with little spoilage evident, may still suffer significant mycotoxin contamination (Manni et al., 2022). Due to the ubiquitous nature of fungal-plant interactions, the contamination of silage with fungal species is considered inevitable and therefore poses a risk to rumen metabolism, animal performance and health (Ogunade et al., 2018b).

The following sections will explore the synthesis of mycotoxins, the current understanding of their relationship with grass silage and potential interactions they may have with the microbiome of the ruminant, which may ultimately impair animal performance. Later sections will explore the detection of mycotoxins, legislation relating to mycotoxin contamination of feedstuffs and the current methods employed in their mitigation.

1.2. An introduction to mycotoxins

1.2.1. Secondary metabolites

Bacteria, plants and fungi all possess the ability to synthesise metabolites of low molecular mass that are classified as not vital for their growth and survival, and so are defined as secondary metabolites (Pang et al., 2021; Sanchez and Demain, 2011). Secondary metabolites can be comprised of fatty acids, sugars and proteins with varied chemical structures, and possess a range of characteristics from pigments to toxicants (Rokas et al., 2020). Secondary metabolites have also been of interest to society, particularly with regard to the medicinal capabilities of some of these compounds (Keller, 2019). The most well-known secondary metabolite was the discovery of penicillin in 1928 by Alexander Fleming (Fleming, 1929; Gaynes, 2017).

If not vital for growth and reproduction, then the biological purpose of synthesising secondary metabolites has puzzled scientists. Secondary metabolites must provide some competitive advantage for the organism, for the pathways of their synthesis to have evolved and been retained (Reverberi et al., 2010; Rokas et al., 2020). With penicillin, the antibiotic effects provide a clear example of cause; a compound to provide *Penicillium* fungi with a competitive advantage over the bacteria in the immediate vicinity, for resources (Ezzat et al., 2007). There are numerous secondary fungal metabolites that can be grouped under antibiotic or antimycotic categories, however there are multiple others that possess no antibiotic or antimycotic properties, and so their purpose has eluded scientists historically. Ciegler, in 1982, wrote:

"*Perhaps the problem lies in attempting to categorize all secondary metabolites into one given function. More likely, secondary metabolites play a variety of roles or, perhaps, at times, no role at all in the struggle of a given microorganism for survival."*

This statement was echoed by O'Brien and Wright, (2011), who suggested that grouping metabolites by their apparent mechanism of action in relation to "intermicrobial warfare", led the historic scientific community to underestimate the true range of secondary metabolites that are synthesised. With the search for novel medicinal compounds and the advent of next generation sequencing, some of the alternative roles of secondary metabolites in the ecosystem were revealed (Venkatesh and Keller, 2019). Rokas et al., (2020) and Sanchez and Demain, (2011) proposed that the main functions of secondary metabolites are 1) competitive "weapons" against other organisms; 2) metal transportation agents; 3) plant-

microbe symbioses agents; 4) sexual hormones; 5) differentiation effectors; 6) quorum sensing agents; and 7) ultra-violet radiation protection.

A number of fungal species have been found to upregulate the production of mycotoxins during periods of oxidative stress (Reverberi et al., 2010) and it is increasingly clear that although secondary metabolites are often considered as “by-products” of a primary metabolic pathway, the complexity of their chemistry and influence on the surrounding environment are not to be underestimated (Rohlfs et al., 2007). This is highlighted particularly with respect to fungal secondary metabolites and their impact on the agriculture and livestock industries.

1.2.2. Mycotoxins

A major impact that fungi in particular can have on their surrounding environment, is the production of mycotoxins (Reverberi et al., 2010; Rokas et al., 2020). Mycotoxins are secondary metabolites that have been shown to exert negative effects on humans and animals and at their most severe, carcinogenic and immunosuppressive activities of some mycotoxins pose a direct threat to livestock health, public health and food security (Hussein and Brasel, 2001; Malekinejad and Fink-Gremmels, 2020). Additionally, the ubiquitous nature of fungi means contamination with mycotoxins can occur at any stage in the feed chain; from the field to the feed, the animal and even carried through to the consumer (Bryden, 2012).

The detrimental effects of mycotoxin contamination were initially brought to attention in the 1960’s with “Turkey X disease” which saw the sudden death of over 100,000 turkey poults in the UK with symptoms of nervous deterioration (Daou et al., 2021; Richard, 2008). Unknown at the time, an import of ground-nut from Brazil for use in feed was later found to be heavily contaminated with the mycotoxin aflatoxin B_1, a metabolite synthesised by *Aspergillus flavus* (Klich, 2007). From the 1960’s onwards in the UK, aflatoxins ascended to the forefront of concern for animal and even human health, spurring investigations further afield into other fungal metabolites that may pose a threat (Pfliegler et al., 2020; Richard, 2008). Alongside emerging research, governing bodies such as the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations (UN) set out to start monitoring and controlling mycotoxin contamination within the global food chain (Jelinek et al., 1989). By 1977, the World’s first Mycotoxin Conference was held, with discussions around aflatoxins, zearalenone (ZEA), ochratoxins, and trichothecenes. All had been proposed for regulation due to their carcinogenic, immunosuppressive or hepatoxic properties on humans and animals (Logrieco et al., 2018).

Today, the JECFA (Joint Expert Committee on Food Additives) is comprised of scientists from the WHO and FAO, who set out guidelines on maximum safe mycotoxin exposure limits for humans and animals worldwide (World Health Organization, 2018). Guidelines are updated when appropriate scientific evidence suggests so, however not all known mycotoxins to date, are included within these guidelines.

1.2.3. Common mycotoxins in forage and grains

Due to the ability of one genera of fungi to synthesise more than one mycotoxin, and the various chemical activities of an individual mycotoxin, grouping mycotoxins solely by their structure or main effects can be challenging (Desjardins, 2006). The main mycotoxins of concern found in forages and grains are synthesised by the fungal genera of *Aspergillus*, *Fusarium, Penicillium, Claviceps* and *Alternaria* (Kos et al., 2023).

1.2.3.1. Aflatoxins

Aflatoxins are considered the most toxic naturally formed carcinogenic agents, and are synthesised by *Aspergillus* species of fungi, with *Aspergillus flavus* being the most studied in agricultural soils (Klich, 2007). Off all the derivative metabolites of aflatoxins, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2 are the most common in agricultural crops, and are found in particular in oilseeds, maize and nuts (Klich, 2007). Aflatoxins are derivatives of difuranocoumarins, containing a furan ring of four carbon atoms and one oxygen atom, attached to a coumarin (loosely described as a benzene ring sharing two carbon atoms with a lactone ring; https://www.ncbi.nlm.nih.gov/, (2024); Figure 1-4).

Aflatoxins have been well studied with regards to the contamination of feed for ruminant animals, in particular their transfer into the milk (Muaz et al., 2022; Rodríguez-Blanco et al., 2020). In mammals, aflatoxin B_1, is metabolised by the CYP450 enzyme to form AFB_1-exo 8,9 epoxide, which forms lesions in DNA and leads to subsequent gene mutations (Benkerroum, 2020). Numerous studies have demonstrated aflatoxin exposure can lead to hepatocellular carcinoma (Benkerroum, 2020), damage bovine leukocytes (Mehrzad et al., 2020), impair bovine spermatozoa (Komsky-Elbaz et al., 2020) result in extreme immunosuppressive activity in dairy cows (Ghadiri et al., 2019), and also pose a substantial risk to the human population through the carryover of the aflatoxin M_1 metabolite into the milk of dairy cows (Ahmadi, 2020; Min et al., 2021). The most heavily regulated mycotoxin worldwide (Klich, 2007), threshold limits for the safe levels of aflatoxin in feed for the EU are set at 0.005 mg/kg DM in compound feed for dairy cattle and calves (European Union (EU), 2006). Though an

important mycotoxin, aflatoxin contamination is rarely observed in grass silages (Manni et al., 2022) and will not be a mycotoxin of major focus in the rest of this thesis.

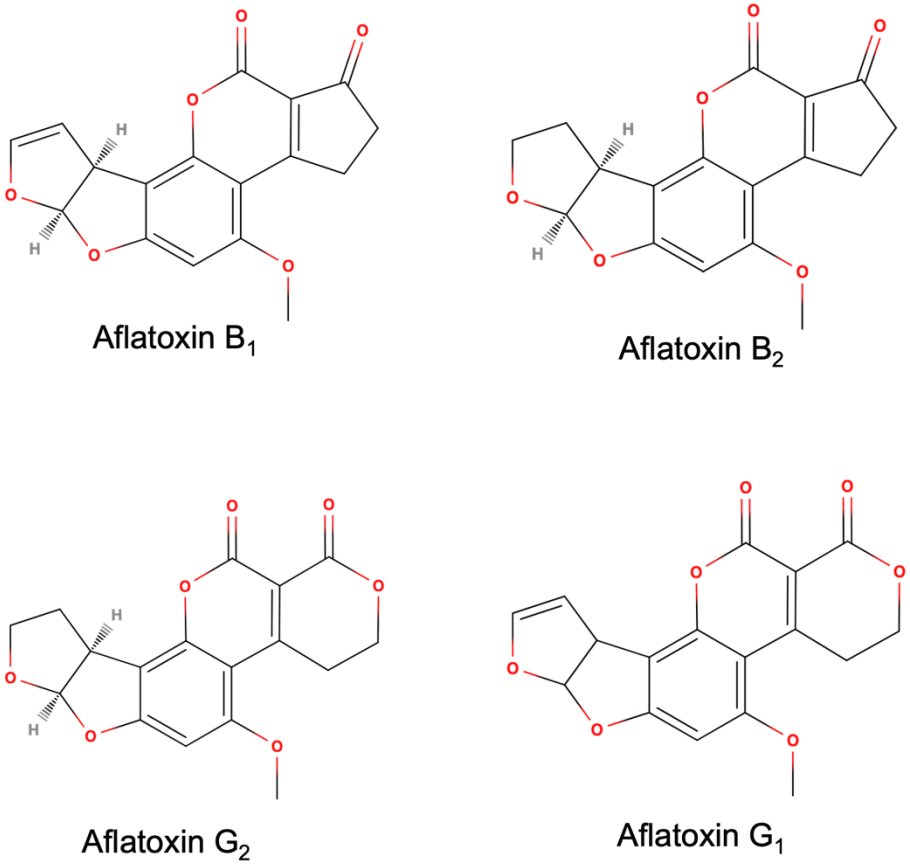


Figure 1-4. The chemical structure for the four common aflatoxin metabolites found in agricultural crops; aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2.

1.2.3.2. Trichothecenes

Trichothecenes are a group of mycotoxins that are synthesised by *Trichothecium, Trichoderma*, *Myrothecium*, and most importantly to agriculture, *Fusarium* (McCormick et al., 2011). Due to the vast ecological niches that these different fungal genera inhabit, over 200 mycotoxins fall under the classification of trichothecenes (McCormick et al., 2011). They are classified as Type A, B, C or type D trichothecenes, dependent on additional functional groups attached to the main tricyclic 12, 13 – epoxytrichothecene structure (Kimura et al., 2007). Most prevalent in agricultural crops, however, are the Type A trichothecenes of neosolaniol, T2-toxin and 15-diacetoxyscirpenol and the Type B trichothecenes of deoxynivalenol (DON), 3-acetyl deoxynivalenol and nivalenol (Figure 1-5).

The main mechanism of action of trichothecenes in animals is the inhibition of the initiation of protein synthesis by binding to the 60S ribosome unit, or by interfering with the elongation and termination phase of protein synthesis (Kiessling, 1986). Infection of grain with trichothecene mycotoxins was held responsible for the cause of alimentary toxic aleukia in Russian populations of the 1930's where the affected experienced diarrhoea, nausea, a decrease in leukoycte numbers (aleukia) and extreme and often fatal haemorrhaging (Bennett and Klich, 2003). Due to the severity of trichothecene action, DON in particular, has been well studied with regards to its impact on livestock. Deoxynivalenol, or vomitoxin, has been found to contaminate corn, wheat, barley and maize and grass silages (Korosteleva et al., 2009) and commonly results in emesis (vomiting) and diarrhoea, which can lead to death.

In dairy cows, DON has been demonstrated to affect metabolism in the rumen, increase ammonia concentration (Dänicke et al., 2005), lead to reduced milk fat yields (Charmley et al., 1993) and cause a depression in neutrophil activity (Korosteleva et al., 2009). Despite this, ruminants are thought to be able to tolerate higher contamination levels of DON than monogastric animals due to the ability of rumen microorganisms to degrade DON into the derivative metabolite de-epoxy deoxynivalenol which is considered relatively less harmful (Debevere et al., 2020a; Kiessling et al., 1984; King et al., 1984) The risk to the food chain through dairy products is therefore considered minimal as studies have demonstrated that DON does not enter the milk of cows exposed to the toxin (Keese et al., 2008b; Prelusky et al., 1984). This is reflected in the European Union recommendation which is set at a DON intake of no greater than 2000 µg/kg daily for dairy calves less than 4 months old in comparison to 500 µg/kg for adult pigs. A recent study by Reyes-Perea et al. (2023) however, suggested that de-epoxy deoxynivalenol may not be as harmless to dairy cows as previously thought, as the metabolite is able to disrupt the activity of ovarian theca cells by instigating cell death. Furthermore, Debevere et al. (2020a) demonstrated that during *in-vitro* fermentation at

a pH lower than 5.8, the ability of the rumen microorganisms to degrade DON was impaired, suggesting that there is still a risk associated with contamination in dairy cow feeds, and that further research is required.

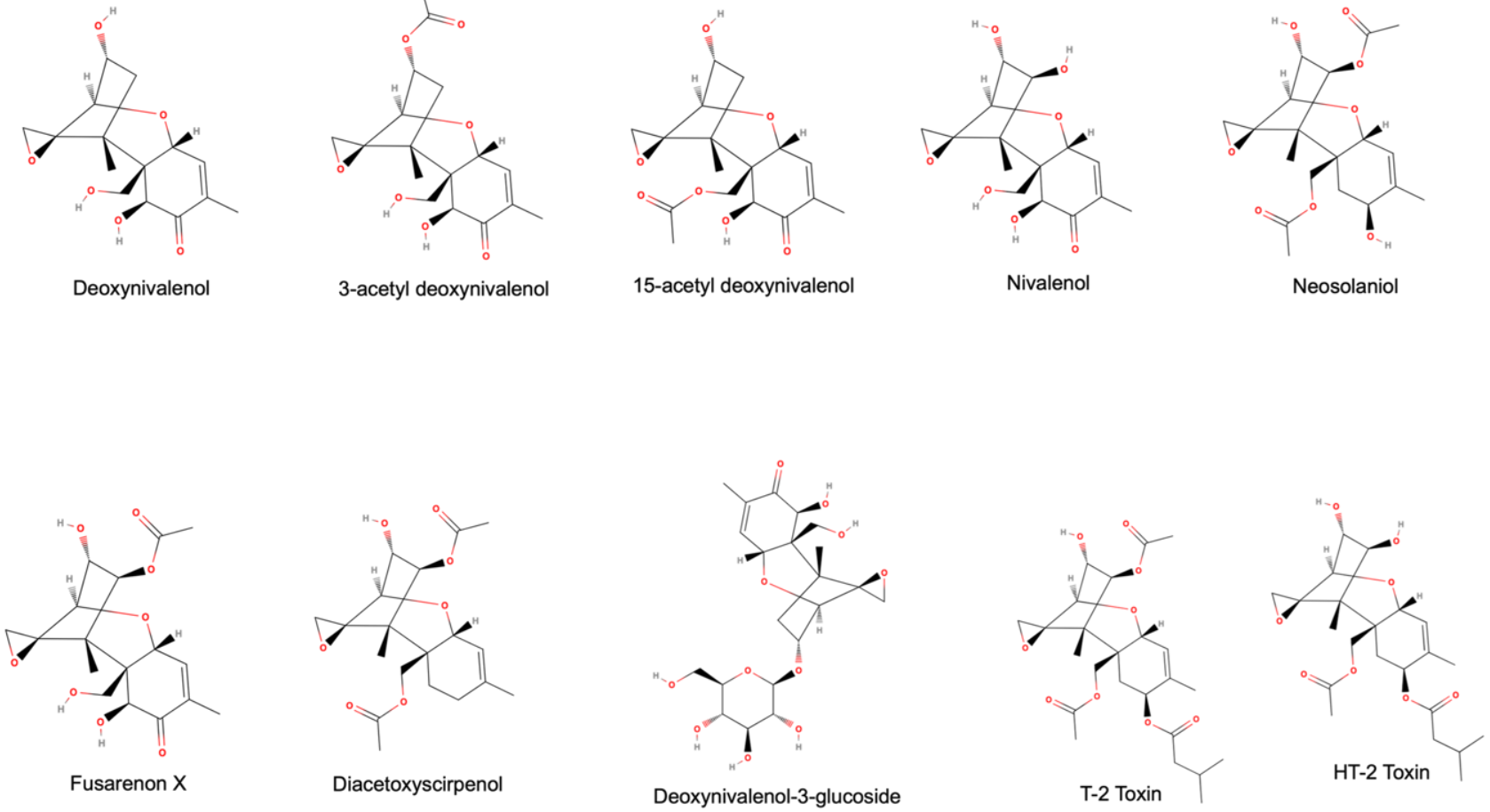
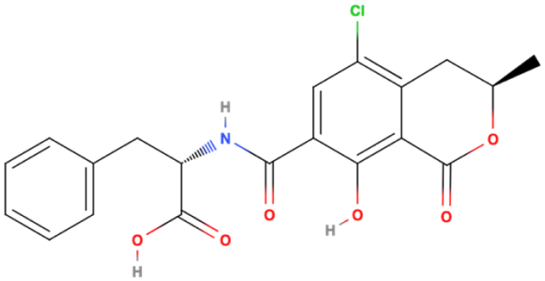


Figure 1-5. The chemical structure for a selection of Type A and Type B Trichothecenes, found in agricultural crops.

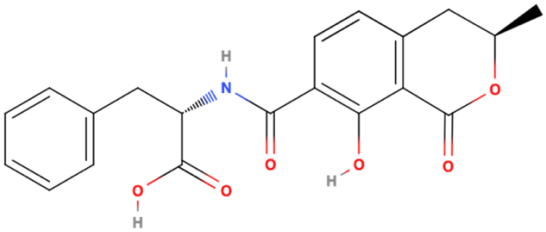
1.2.3.3. Ochratoxins

Ochratoxins are a group of mycotoxins that are synthesised by *Penicillium* and *Aspergillus* fungi and have been labelled as immunosuppressive and carcinogenic (Tao et al., 2018). There are numerous derivative metabolites of ochratoxins but the most common in agricultural crops involves ochratoxins A and B which often contaminate cereals and their products such as bread (Tao et al., 2018). Structurally, ochratoxins are isocoumarins, coupled with beta-phenylalanine, where ochratoxin B is the dechlorinated version of ochratoxin A (Pratt-Hyatt, 2020; Figure 1-6).

Ochratoxins have been demonstrated as nephrotoxic in mammals and possess the ability to degrade mRNA coding for phosphoenolpyruvate carboxykinase in the kidney (Kiessling, 1986). Despite the lack of human studies or conclusions on the precise mechanism of action, ochratoxin A has been classified as carcinogenic to humans due to its ability to exert oxidative damage on DNA (Pfohl-Leszkowicz and Manderville, 2007), and has been reported to be responsible for the growth of tumours in mice when exposed (El Khoury and Atoui, 2010). Immunosuppressive activity has also been demonstrated by ochratoxin A, through the inhibition of T lymphocyte IL-2 receptor expression (Lea et al., 1989) and it is thought that ochratoxin A may disrupt bacterial protein synthesis, through interference with the aminoacylation of tRNA (Kiessling, 1986). Acute ochratoxin exposure has also been associated with brain, liver, kidney and heart haemorrhage in small non-ruminant mammals, however there is evidence to suggest that ruminants are able to degrade ochratoxin A into the less toxic metabolite ochratoxin-alpha (Hult et al., 1976; Kiessling et al., 1984). Despite this, a study by Blank et al., (2003) suggested that the capacity to degrade ochratoxins by rumen microorganisms can vary, with some accumulation of ochratoxin A in the blood serum of sheep that were exposed to levels as low at 9.5 µg/kg ochratoxin A per kg of sheep body weight.



Ochratoxin A



Ochratoxin B

Figure 1-6. The chemical structure for ochratoxin A and ochratoxin B, found in agricultural crops.

The occurrence of ochratoxins is more common in maize silages (Queiroz et al., 2018; Tangni et al., 2013), and farm studies to date have not identified ochratoxin A as a prominent contaminant of grass silages (Manni et al., 2022; McElhinney et al., 2016a; Tangni and Van Hove, 2013). As a result, ochratoxins will not feature heavily in this thesis.

1.2.3.4. Ergot alkaloids

Ergot alkaloids are a class of mycotoxins produced by the *Claviceps* genus of fungi and are associated with ergot contamination of cereals (Schiff, 2006). Ergot alkaloids, include derivative metabolites such as ergotaminine, ergosinine, methylergovaline, and lysergic acid (Figure 1-7) however *Claviceps* species have been found to synthesise approximately 80 different ergot alkaloids in total (Schiff, 2006). In humans, ergot alkaloids were responsible for the illness St Anthony's fire, that caused people to experience extreme burning sensations and serious convulsions, whilst extremities often became gangrenous (Grzybowski et al., 2021). Ergot alkaloids have been of great interest to medicine through time due to their mixed and variable properties. In 1528, preparations of ergot were purportedly used by midwives to induce uterine contractions during labour (Schiff, 2006), and present day uses include the controversial investigations into lysergic acid (a precursor to lysergic acid diethylamide; LSD) as therapeutic agents for mental health conditions such as severe depression (Dyck, 2015; Fuentes et al., 2020).

Primarily, ergot alkaloids are central and peripheral nervous system effectors (Schardl et al., 2006) due to their structural similarity to the neurotransmitters serotonin, dopamine, and adrenaline (Gerhards et al., 2014). Chemically, they are indole alkaloid compounds, comprised of tetracyclic ergoline ring including various additions of functional groups which modify the biological effect of the compound (Gerhards et al., 2014).

In livestock, the response to ergot alkaloid exposure is highly variable and can range from similar gangrenous impact on extremities as in humans, to decreases in feed intake, reduced milk production, hyperthermia and impaired fertility, that may not always be immediately identified as a result of ergot exposure (Klotz, 2015; Schrenk et al., 2024). There is therefore no recommended maximum safe level established by the EU for ruminant consumption, however in humans ergot alkaloids in milling products of barley and oats (ash content < 900 mg/ 100g DM) was reduced to 50 µg/kg in July 2024 (European Union (EU), 2024). A study by Wolff (2005) described in Schrenk et al. (2024) demonstrated that ergot alkaloids are unlikely to transfer through into the milk of dairy cows upon oral exposure in high enough concentration to cause issue to human health.

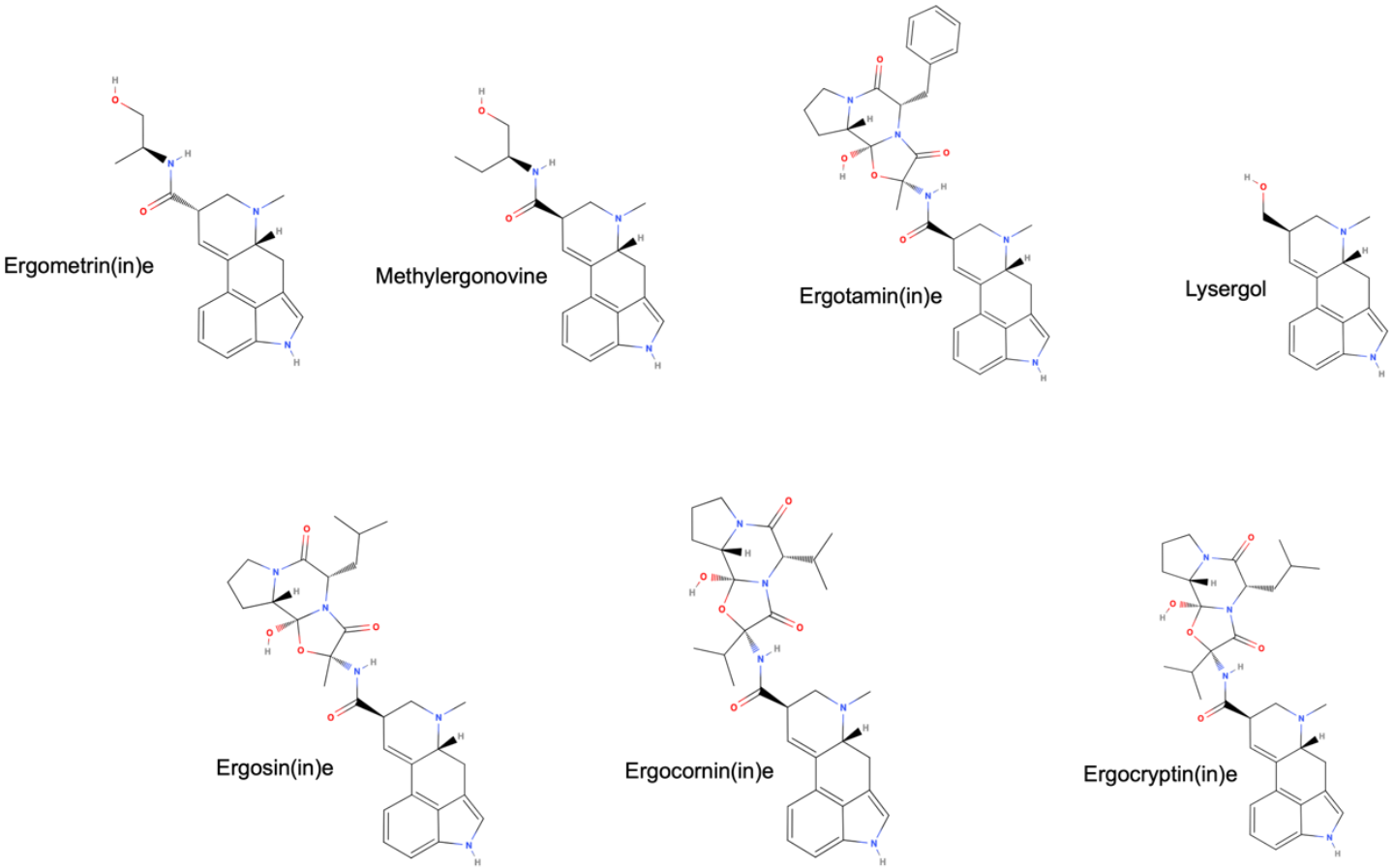
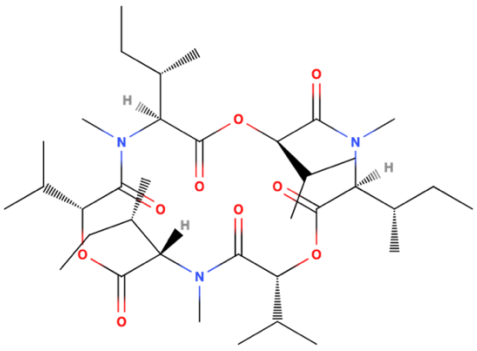


Figure 1-7. The chemical structure of a number of ergot alkaloids, found in agricultural crops.

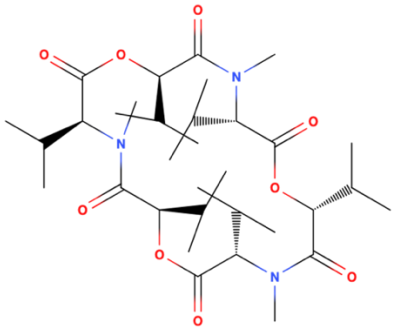
1.2.3.5. Enniatins

Enniatins are a group of *Fusarium* mycotoxins that demonstrate a wide range of activity including antifungal, antibiotic and insecticidal activity (Korre et al., 2017). Structurally they are cyclo-hexadepsipeptides and most commonly occur in agricultural crops such as cereal grains, as enniatins A, A_1, B and B_1, with enniatin B_1 the most studied (Figure 1-8; Juan-García et al. (2013)). They display ionophoric activity, where they have a high affinity for cations which can disrupt normal processes within a cell by altering intracellular concentrations of these ions (Korre et al., 2017). Studies have demonstrated cytotoxic activity of enniatins on mammalian cells with one method of ionophoric activity on K^+ ions leading to disruption of mitochondrial activity and eventual apoptosis (Tonshin et al., 2010).

Enniatins have also been demonstrated to induce oxidative stress on cells, inhibit acyl-CoA cholesterol acyl-transferase and have been reported to exhibit estrogenic activity. Their antibiotic activity has been demonstrated effective against *E. coli, Listeria monocytogenes,* and *Staphylococcus aureus* strains and their antifungal activity acts against *Beauveria bassiana* and *Trichoderma harzianum* (Korre et al., 2017). In dairy cows, the rumen microbial organisms have demonstrated the ability to degrade enniatin B_1, but few other studies on enniatin impacts exist (Debevere et al., 2020a). In monogastric animals, pathways of metabolism of enniatins vary greatly dependent on species and the subsequent metabolites produced are mostly uncharacterised (Křížová et al., 2021). Due to the little research that has been carried out *in-vivo*, the EU have been unable to set limits through lack of information for an adequate risk assessment.



Enniatin A/A_1



Enniatin B/B_1

Figure 1-8. The chemical structure of enniatin A/A_1 and enniatin B/B_1, found in agricultural crops.

1.2.3.6. Fumonisins

Fumonisins (FUM) are mainly produced by *Fusarium* species of fungi and comprise 28 structurally related metabolites of which fumonisin B_1, B_2, and B_3 are most common (de Oliveira et al., 2014) in agricultural products, such as maize. Fumonisins (Figure 1-9) are diesters and are structurally similar to sphingolipids, with a 19-20 carbon chain aliphatic acid backbone (Pitt, 2014). Fumonisin B_1 can inhibit ceramide synthase, which prevents the conversion of sphingosine to dihydroceramide and ceramide, leading to an accumulation of sphingosine (Blank et al., 2005) .This causes disruption to the cell membrane integrity, which hosts surface receptors and ion pumps, resulting in apoptosis (Pitt, 2014). Some studies have also suggested FUM exposure can produce reactive oxygen species, that induce DNA damage and an increase in cytotoxicity has been noted when in present in combination with aflatoxin B_1; demonstrating additive affects (J. Chen et al., 2021). Others have demonstrated FUM induced reproductive damage in animals through impairing the survival of oocytes and interfering with progesterone production (J. Chen et al., 2021). In horses, FUM contaminated feed is responsible for leukoencephalomalacia, where symptoms include extreme lethargy, anorexia, blindness and seizures, commonly caused by haemorrhage and malacia of the brain, ending in death (Vendruscolo et al., 2016). Ruminants are thought to be more tolerant of FUM exposure than other livestock, but the mechanisms behind this are unknown. Caloni et al. (2000) suggested that fumonisin B_1 is not well metabolised by rumen microorganisms (only 12-18 %) and therefore rumen detoxification is not a factor in the lower sensitivity displayed by ruminants. Additionally, Spotti et al. (2001) found no evidence for metabolism in the liver of cattle either. The EU regulation sets out guidance values for fumonisins B_1 and B_2 in adult ruminants as no greater than 50 mg/kg in compound feed (European Union (EU), 2006).

Fumonisins have in most cases been assessed alongside other common *Fusarium* mycotoxins in studies on cattle performance, due to the common occurrence of co-contaminant mycotoxins ZEA and DON when FUM are present, particularly in maize. Due to mycotoxin interactions, this makes it challenging to identify the influence of FUM only on cattle health and performance but does mean conclusion from the studies are more applicable to on-farm. A study where FUM were the major contaminant out of a combination, DMI of steers was reduced, and rumen pH was reduced also with exposure (Batista et al., 2024). Similarly, Duringer et al. (2020) also demonstrated a reduction in rumen pH, with a diet of 1.7 mg/kg TMR DON and 3.5 mg/kg TMR FUM. Catellani et al. (2023) demonstrated a lower milk yield and feed efficiency in dairy cows fed a TMR including DON at < 1000 µg/kg, ZEA at < 260 µg/kg and FUM at < 280 µg/kg.

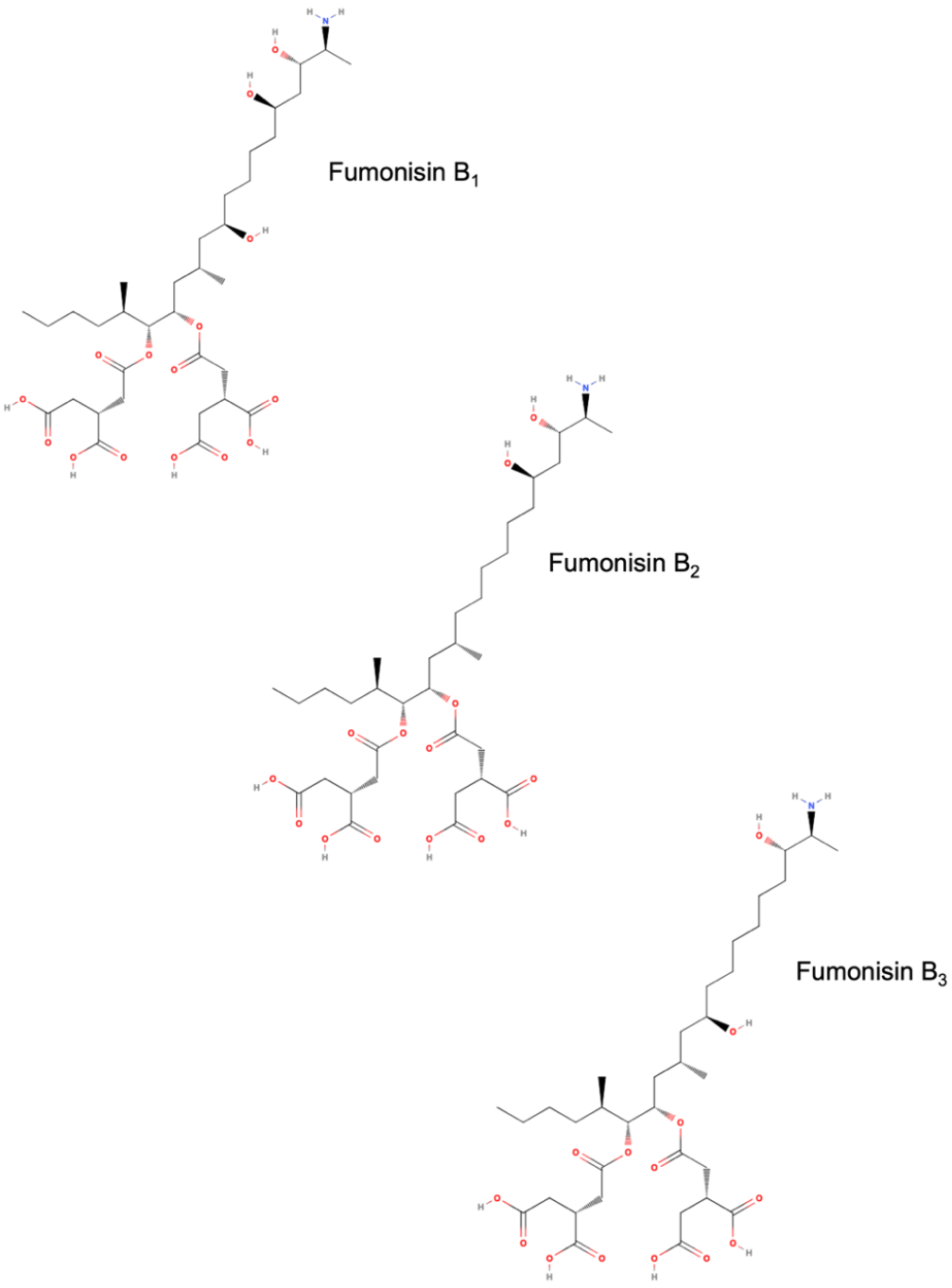
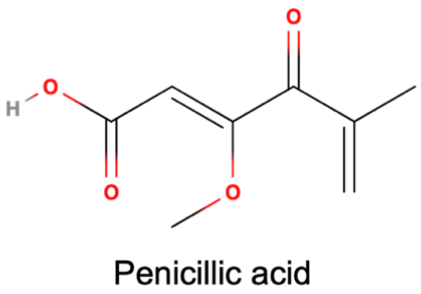


Figure 1-9. The chemical structure of fumonisins B_1, B_2 and B_3, found in agricultural crops.

1.2.3.7. Penicillic acid

Penicillic acid (PEN) has been demonstrated as an antibiotic and antibacterial agent against both gram-positive and gram-negative bacteria (Geiger and Conn, 1945; Raphael, 1947) with the ability to inhibit quorum sensing by bacteria such as *Pseudomonas aeruginosa* (Rasmussen et al., 2005). Penicillic acid also displays antifungal activity against phytopathogenic fungi such as *Phytophthora cactorum* (Kang and Kim, 2004). A lactone mycotoxin (Figure 1-10), it is a derivative of the antibiotic penicillin but has inhibitory effects on different bacterial species (Ezzat et al., 2007). It can be produced by species of *Aspergillus* and *Penicillium* fungi (Frisvad, 2018) and co-exposure with ochratoxin A has been demonstrated to work synergistically with regards to hepatoxicity in mice (Sansing et al., 1976).

In livestock PEN has demonstrated antidiuretic and vasodilatory properties and is reported to be cytotoxic (Frisvad, 2018) but its level of oral toxicity is considerably lower than other mycotoxins such as DON, and aflatoxin B1 (Bianchini and Bullerman, 2014); as such, the impact of PEN on cattle has been a neglected area of study. Furthermore, there are no EU guidance limits for PEN concentration in feed for livestock. Penicillic acid has been reported in maize (Munkvold et al., 2019), but grass silages contaminated with *Penicillium* able to produce PEN have been identified (O'Brien, 2010; O'Brien et al., 2008).



Penicillic acid

Figure 1-10. The chemical structure of penicillic acid, found in agricultural crops.

1.2.3.8. Mycophenolic acid

Mycophenolic acid (MPA) is utilised in human medicine as an immunosuppressant under the brand name Mofetil; mycophenolate being the active ingredient (Bentley, 2000) as it has higher bioavailability than MPA (Mohr et al., 2007). Structurally, it is a class 2-benzofuran (https://www.ncbi.nlm.nih.gov/, 2024) and is an inhibitor of the enzyme inosine monophosphate dehydrogenase, which is involved in the synthesis of guanine (Routledge and Hutchings, 2013). As T and B lymphocytes are uniquely dependent on this pathway for cell proliferation, MPA inhibition leads to a suppression in the immune response.(Hiemstra and Jayne, 2009)

Mycophenolic acid has been identified in grass silages by Schneweis et al. (2000) and is common alongside the other *Penicillium* mycotoxins roquefortine C and PEN (Gallo et al., 2015; O'Brien, 2010). There is only one known study to date of the effect of MPA exposure on a ruminant *in-vivo* (Gallo et al., 2015), by Mohr et al. (2007) who investigated the effect of MPA on health parameters of sheep with no effects on exposure found on liver metabolism, body temperature, serum enzymes or total bilirubin concentration after administering a maximum of 300 mg/MPA per sheep daily for 44 d. Owing to the lack of information available there are no recommendations set out by the EU for levels of MPA in feed for livestock.

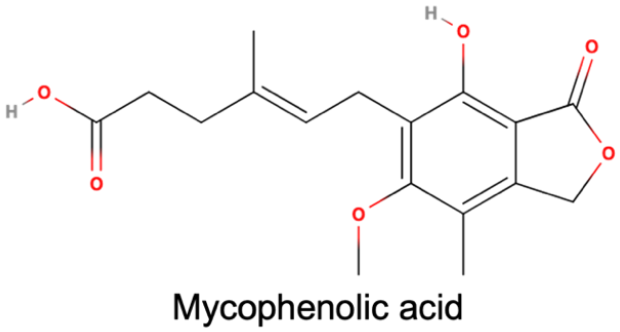


Figure 1-11. The chemical structure of mycophenolic acid, found in agricultural crops.

1.2.3.9. Fusaric acid

Fusaric acid (FUS) is mycotoxin produced by species of *Fusarium* fungi and is a derivative of picolinic acid. Chemically it is a polyketide metabolite (Figure 1-12), and carboxylic acid (https://www.ncbi.nlm.nih.gov/, 2024; Niehaus et al., 2014). It is antimicrobial (Bacon et al., 1996), can disrupt quorum-sensing (Tung et al., 2017), has the ability to penetrate cell membranes and is a metal—chelating agent (Arumugam et al., 2021). It is able to form conjugates with zinc, iron, copper and manganese with the potential to disrupt biological processes reliant on these ions (Arumugam et al., 2021). Fusaric acid has been reported in maize where it often co-occurs with other *Fusarium* associated mycotoxins of DON and ZEA (Dänicke et al., 2005).

In animals fusaric acid can induce downregulation of the expression of several proteins involved in mitochondrial biogenesis (Arumugam et al., 2021). Additionally, Wang and Ng (1999) reported that fusaric acid contaminated feed, ingested by rats was transferred through the milk to the young, causing a reduction in weight gain and interfered with serotonin and tyrosine activity in the brains of the offspring. The synergistic effect of FUS and DON has also been reported on pigs, which led to a reduction in weight gain (Wang and Ng, 1999). The effect of fusaric acid on dairy cow performance and health has not been well studied, with only one study to date carried out *in-vitro* by May et al. (2000) who demonstrated the ability of FUS to inhibit the growth of two prominent rumen microorganisms, the fibrolytic bacteria *Ruminococcus albus,* and the methanogenic archaea *Methanobrevibacter ruminantium.* Despite the ability of FUS to impact on rumen microbial community composition, and its ability to act on neurotransmitter activity, there are no guidance values set out by the EU on FUS contamination of feed.

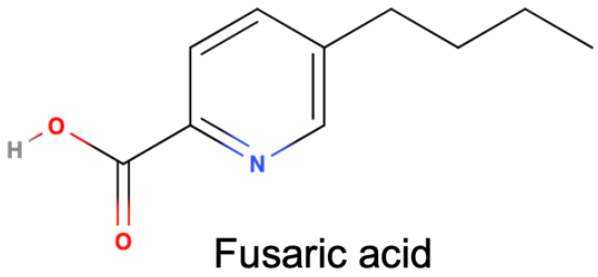


Figure 1-12. The chemical structure of fusaric acid, found in agricultural crops.

1.2.3.10. Zearalenone

Zearalenone is a mycotoxin synthesised by species of *Fusarium* and is well studied with regards to its negative impact on the health and performance of livestock (Minervini and Aquila, 2008) . Found to commonly occur in maize and grains, it has oestrogenic activity due to the similar structure it shares with 17β-estradiol (Bulgaru et al., 2021). Chemically it is a lactone mycotoxin (Figure 1-13) and can resist high temperatures and ultra-violet radiation (Bulgaru et al., 2021).

The primary mechanism of action of ZEA in dairy cows is impaired fertility, hyperoestrogenism, (Minervini and Aquila, 2008) and reduced milk production (McKay et al., 2019), however impacts on rumen fermentation have also been reported. Rivera-Chacon et al. (2024) reported a reduction in valerate proportion in the rumen, following ZEA administered at 9.45 mg/cow/d in lactating Simmental cows, and Hartinger et al. (2022) demonstrated a lowered total short-chain fatty acid concentration in Holstein dairy cows when exposed to a TMR contaminated with 5 mg/kg DM ZEA and 20 mg/kg DM FUM. Two prominent rumen bacterial families *Prevotellaceae* and *Lachnospirachaea* associated with protein utilisation and in the case of the latter, also ammonia production (Seshadri et al., 2018), were found to reduce in relative abundance with ZEA and FUM addition in the same study (Hartinger et al., 2022).

The EU sets out guidance limits for ZEA at 0.5 mg/kg (DM at 88 %) for calves, dairy cattle and sheep (European Union (EU), 2006) and although important to dairy cow health and performance, ZEA is more commonly found in maize silages than grass silage (De Mulder et al., 2017) and so will not feature heavily in this thesis.

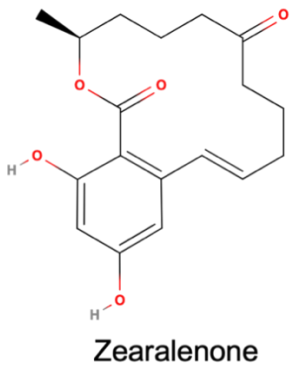


Figure 1-13. The chemical structure of zearalenone, found in agricultural crops.

1.3. An introduction to the rumen microbial ecosystem

The ruminant digestive system is specifically adapted for the breakdown of fibrous plant matter, due to the symbiotic relationship it shares with the fermentative microorganisms that inhabit the rumen (Membrive, 2016). The largest digestive chamber of a total of four, the rumen is comprised of a range of bacteria, archaea, fungi, protozoa and viruses, forming a complex ecosystem within which numerous microbial metabolic processes are ongoing at any one point (Millen et al., 2016). The particular composition, species and strains of microorganisms present, are heavily influenced by genetics, environment and diet of the host animal (Newbold and Ramos-Morales, 2020), though there exists a “core” microbiome across all ruminant species (Henderson et al., 2015). Henderson et al. (2015) reported that the core microbiome consists of less than 0.25 % of the overall species identified in the rumen, but the high abundance of these core groups represents between 30 and 60 % of the overall microbiome.

1.3.1. The liquid phase, solid phase and rumen epithelial wall communities

The rumen ecosystem can be approximately split into three separate sections of ecological niche; the solid phase, liquid phase and rumen epithelial wall communities (De Mulder et al., 2017). The solid phase includes a mat of digesta located dorsally, where contractions of the rumen wall conglomerate the feed into a large, rounded mass that spans the width of the rumen and includes a high concentration of cellulolytic bacteria that are attached directly to the feed particles, or in a biofilm (Membrive, 2016). Fungi are also present, with networks of hyphae growing across the digesta mat (Windham and Akin, 1984). Protozoa have also demonstrated ability to catabolise fibrous compounds and so are found in amongst the solid phase digesta also (Williams and Coleman, 1997).

The liquid phase is a ventrally-located suspension comprised of the end-products and organic acids of bacterial fermentation, gases such as ammonia, methane, H_2 and CO_2 in various stages of solubility, free bacteria, ciliate protozoa, fungi and archaea (Nagaraja, 2016). Rumen contractions carried out by the host animal allows for the liquid phase to be washed over the mat of digesta in the solid phase, encouraging fermentation of the feed (Membrive, 2016). Small boluses of partially digested feed enter the reticulum for mechanical breakdown by the grinding movement of the ruminant molars. This also increases the surface area of the feed for bacterial attachment, before re-entering the rumen, the omasum, and then abomasum for acid digestion, as in monogastric stomachs (Membrive, 2016). Gases (majority methane) are expulsed from the rumen via the oesophagus and out of the ruminant via a process known as eructation (Membrive, 2016).

The rumen epithelial wall is covered on the internal surface in projections known as papillae, with a honeycomb structure where a series of fungi, archaea, and bacteria inhabit the space (referred to as the epimural community; (De Mulder et al., 2017; Membrive, 2016). Oxygen scavenging and ureolytic bacteria and archaea have been demonstrated to preside over this niche (De Mulder et al., 2017).

1.3.2. The rumen microbial community

1.3.2.1. The rumen bacteria

The rumen bacteria comprise the greatest proportion of the microbial rumen community and can reach an abundance of up to x 10^{10} CFU per g of digesta consumed (McSweeney and Mackie, 2012). Bacteria of the rumen can be generalists or specialists with their primary substrates ranging from cellulose to starch, to pectin, with some able to hydrolyse lipids and proteins (Seshadri et al., 2018); Table 1-3). They are the main producers of the volatile fatty acids (VFAs) of acetate, propionate and butyrate, proportionally formed in that order, that are utilised by the dairy cow and as such the manipulation of the bacterial population of the rumen has garnered extensive interest in the potential for improving the performance of dairy cows. Diet and genetics are able to influence the microbial composition (Deusch et al., 2017; Henderson et al., 2015; Russell and Rychlik, 2001), and the former has been extensively studied with respect to manipulating the fermentation processes to improve feed use efficiency, or to reduce the availability of end products for methanogenesis in the efforts to reduce the climatic impact of agriculture (Belanche et al., 2012; Morgavi et al., 2010).

The most dominant phyla of the rumen microbiome include the Bacteriodetes and Firmicutes, followed by the Proteobacteria. Bacteroidetes are mostly comprised of families involved in the utilisation of proteins, starches and available sugars, whilst Firmicutes are most associated with fibre degradation. Notable families of Firmicutes include Ruminococcaceae and Lachnospiraceae, and prominent rumen genera identified of both Bacteroidetes and Firmicutes include *Prevotella, Ruminococcus,* and *Butyvibrio* (Henderson et al., 2015). The main bacterial families identified and their substrate utilisation and common end-products of their fermentation as described by Seshadri et al. (2018) are listed in Table 1-3.

Numerous studies have set out to target and identify individual members of the rumen bacterial community, originally through culturing techniques, but more recently through molecular methods (i.e. The Hungate 1000 project).

1.3.2.2. The rumen archaea

A large proportion of archaea (phylum: *Euryarchaeota*) in the rumen are the methanogens, which include genera such as the most prominent *Methanobrevibacter*, *Methanomicrobium,* and the less abundant genus *Methanosarcina* (Janssen and Kirs, 2008). Numerous studies utilising molecular techniques to target genes encoding for the 16S rRNA subunit, or methanogen specific genes such as those encoding for methyl co-enzyme reductase (mcrA) (Morris et al., 2014), have aimed at elucidating the composition of methanogenic archaea in the rumen with contrasting and often inconclusive results (Janssen and Kirs, 2008). There is as a result, a large group of methanogenic archaea that are currently largely unclassified – a study by Tan et al. (2021) found that of the methanogenic community identified on the epithelial wall of the rumen, 80 % were unclassified rumen archaea. They can be of rod, coccoid or filament form and with or without motility (Hook et al., 2010).

Hydrogenotrophic methanogenesis is the most common pathway utilised by the archaea in the rumen, though methane production through other pathways also occurs, namely methylotrophic and acetoclastic methanogenesis (Henderson et al., 2015; Janssen and Kirs, 2008). Acetoclastic methanogenesis is thought however to occur less due to the rapidity of which acetate diffuses across the rumen epithelial membrane in comparison to the growth rate of acetate utilising methanogens (Janssen and Kirs, 2008). Furthermore *Methanosarcina* sp. are the only archaea known to carry out acetoclastic methanogenesis in the rumen and as previously mentioned are at a much lower relative abundance to the more prevalent hydrogenotrophic methanogens of the *Methanobrevibacter* genus (Henderson et al., 2015). Methanogenic archaea are known to associate with protozoa of the rumen such as *Entonidium, Polyplastron* and *Epinidium*, as well as rumen fungi such as *Neocallimastix frontalis* in which they carry out hydrogen transfer (Hook et al., 2010) forming a symbiotic relationship. A study by Levy and Jami (2018) suggested that the proportion of methanogenic archaea associated with protozoa are of a greater abundance in comparison with the free-living archaea present in the rumen. Additionally, archaea of the genus *Methanobrevibacter* have been identified as the predominant archaea that form associations with protozoa whereas methylotrophic archaea such as *Methanomassillicoccacea* sp. were found to be more common in the free-living community (Levy and Jami, 2018).

Table 1-3. A selection of the main prokaryotic phyla identified in the rumen Hungate1000 collection, as detailed by Seshadri et al. (2018), and the substrates utilised by species of each family/order and the main end-products of their fermentation

Phylum	Family/order	Substrate used	Fermentation products
Actinobacteria	Actinomycetaceae	XY, ST, LA, PT	AC, FO, LA, SU
	Bifidobacteriaceae	ST	AC, LA
	Propionibacteriaceae	ST, LA, PT	AC, PR
Bacteroidetes	Bacteroidales	XY, PE, ST, PT	AC, SU
	Bacteroidaceae	XY, PE, PT	AC, PR, SU
	Prevotellaceae	XY, PE, ST, PT	AC, PR, SU
Fibrobacteres	Fibrobacteraceae	CE, XY, PE	AC, FO, SU
Firmicutes	Clostridiales	XY	NH_4GEN, AC, BU, LA
	Acidaminococcaceae	SU	AC, BU, PR
	Bacillaceae	CE, XY, PE, ST, PT	AC, BU, LA
	Clostridiaceae	XY, PE, ST, LA, LI, PT	AC, BU, PR, FO, LA
	Enterococcaceae	XY, PE	PR, LA
	Erysipelotrichaceae	PE, PT	AC, BU, LA
	Eubacteriaceae	LA	ACGEN, AC, BU, PR
	Lachnospiraceae	CE, XY, PE, ST, LA, PT	NH_4GEN, ACGEN, AC, BU, PR, FO, LA
	Lactobacillaceae		LA
	Peptostreptococcaceae	LA	NH_4GEN, ACGEN, AC, BU, FO, LA
	Ruminococcaceae	CE, XY, PE, ST, PT	AC, BU, FO, LA, SU
	Staphylococcaceae		LA
	Streptococcoaceae	XY, PE, ST, PT	LA
	Veillonellaceae	PE, ST, LA, SU, LI	AC, BU, PR, LA
Proteobacteria	Desulfovibrionaceae		AC, FO
	Enterobacteriaceae	ST	AC, PR, FO, LA
	Pasterurellaceae	ST	SU
	Succinivibrionaceae	ST	AC, SU
Synergistetes	Synergistaceae		AC, PR
Spirochaetes	Spriochaetaceae	XY, PE, ST	AC, FO, LA,SU
Euryarchaeota	Methanomassillicoccales	METHYL	CH_4
	Methanobacteriaceae	H_2	CH_4
	Methanomicrobioaceae	H_2	CH_4
	Methanosarcinaceae	ACETO, H_2, METHYL	CH_4

XY = xylan, CE = cellulose, ST = starch, PE = pectin, LA = lactate, PT = protein, FO = formate, AC = acetate, PR = propionate, BU = butyrate, NH_4GEN= ammonia production, LI = lipids, METHYL = methylotrophic methanogenesis, ACGEN = acetogenesis, H_2 = hydrogenotrophic methanogenesis, ACETO = acetoclastic methanogenesis, CH_4 = methane.

1.3.3. The rumen fungal community

The rumen fungi are members of the phylum Neocallimastigomycota and are able to produce a range of ligno-cellulolytic enzymes resulting in fermentation end products of H_2, CO_2 and even VFAs (Li et al., 2021). Furthermore, their hyphae are able to break through lignified cell walls, aiding in further breakdown by other rumen organisms (Bhagat et al., 2023). There have been 10 genera that have been isolated and identified to date, and these are the *Neocallimastix, Orpinomyces, Cyllamyces, Piromyces, Caecomyces, Anaeromyces, Buwchfawromyces, Oontomyces, Pecoramyces* and *Feramyces* (Cheng et al., 2018).

The rumen fungi lifecycle involves two stages, the zoospore and vegetative thallus stage (Bhagat et al., 2023). During the first stage the fungal zoospores are free-floating in the rumen fluid and move toward soluble carbohydrates in the fluid by chemotaxis, before they eventually attach to plant matter, and begin to enter a vegetative state (Akin and Borneman, 1990). This includes a loss of the flagellum, and rhizoid growth occurs through the plant matter, breaking apart plant cell walls (Akin and Borneman, 1990). After attachment and establishment on the plant matter that has entered the rumen, the fungi are able to carry out mitosis, eventually releasing zoospores back into the rumen fluid to begin the cycle again (Bhagat et al., 2023). This cycle is reported to take between 8 to 32 h and plant matter entering the rumen is estimated to be colonised by fungi by 2 h post ingestion (Bhagat et al., 2023).

The carbohydrate active enzymes identified to be produced by rumen fungi include numerous enzymes of the groups: cellulase, hemicellulase, pectinase, amylase and chitinase (Bhagat et al., 2023; Cheng et al., 2018) and an *in-vitro* study reported that up to 62 % of DM provided was degraded by rumen fungi in the absence of bacteria suggesting they are capable of a large proportion of fibrous degradation in the rumen (Windham and Akin, 1984).

Rumen fungi form symbiotic relationships alongside methanogenic archaea in the rumen, with notable relationships including *Methanobrevibacter* and *Piromyces* species (Cheng et al., 2018) as the rumen fungi are able to produce H_2, CO_2, formate, acetate, and lactic acid which are utilised in methanogenesis (Cheng et al., 2018). Additionally, co-culturing of methanogenic archaea and rumen fungi in the laboratory has been shown to increase the diversity of methanogens, suggesting that the rumen fungi also produce unknown factors essential for certain methanogenic species growth (Cheng et al., 2018).

1.3.4. The rumen protozoa

The rumen protozoa are difficult to culture outside of the rumen environment, and as such their contribution to the overall fermentation within the rumen ecosystem is still unclear

(Williams and Coleman, 1997). They comprise two morphologically distinct groups, the holotrichs and entodiniomorphs, with lower classifications of *Isotricha* and *Dasytricha*, for holotrichs and *Ostraconidium, Diploplastron, Entodinium, Eudiplodinium,* and *Epidinium* for entodiniomorphs (Williams and Coleman, 1997; Williams et al., 2020). Microscopy methods were historically used to classify protozoa based upon their morphology, such as size, shape, the presence of a micro or macronucleus and external spines, but this was found to pose substantial challenges as the morphology of certain protozoa was altered under *in-vitro* conditions (Newbold et al., 2015). More recently, studies with 18S rRNA sequencing and accompanying metagenomics analysis have aimed to better understand the role of protozoa in the rumen (Elekwachi et al., 2017; Wang et al., 2019; Williams et al., 2020).

Horizontal gene transfer from bacteria to protozoa has been reported, with the exchange of enzymes relevant for carbohydrate metabolism and as such possess the ability to synthesise polysaccharide lyases, deacetylase, xylanase, and enzymes with pectinase, mannase and chitinase activity (Ricard et al., 2006). Chitinase activity has been hypothesised to be utilised in the predation of fungi by protozoa (Williams et al., 2020). The full extent to which the rumen protozoa participate in the degradation of fibrous matter remains to be evaluated (Williams et al., 2020). However, due to their association with methanogenic archaea, antiprotozoal strategies for use in dairy cow diets have garnered interest as potential methods for the mitigation of methane emissions (Hegarty, 1999; Regensbogenova et al., 2004; Wang et al., 2017).

1.4. The effect of mycotoxins on the ruminant

Most animal studies concerning the effect of mycotoxins on ruminants have been focussed on the most harmful of mycotoxins - aflatoxin B_1 (AFB_1) on the health of the animal, and its ability to enter milk destined for human consumption (Fink-Gremmels, 2008; Flores-Flores et al., 2015). Secondary to aflatoxins, the effects of DON and ZEA have also been investigated but with regards to their impact on the health of cattle, due to the primary mechanisms of these metabolites on the immunity and fertility of mammals (Bulgaru et al., 2021; Duringer et al., 2020; Minervini and Aquila, 2008). However, these mycotoxins are found at lower contamination levels in grass silages (McElhinney et al., 2016a; O'Brien et al., 2006; Penagos-Tabares et al., 2022) and as such, most animal studies have focussed on contamination of concentrates in a total mixed ration (TMR), such as wheat and grains (Sarich et al., 2021; Seeling et al., 2006, 2005). Furthermore, where silage has been considered, it has often been with respect to maize silage (Dänicke et al., 2017; Korosteleva et al., 2009; Simion et al., 2010), which in general presents higher levels of natural contamination of DON, FUM and ZEA in comparison to grass silages (Dänicke et al., 2020). Grass silages have also not been found to contain aflatoxin B_1 at detectable levels (Manni et al., 2022). Despite the primary mechanisms of these metabolites interfering with protein synthesis (DON), fertility (ZEA) and resulting in genetic mutations (AFB_1), there is evidence to suggest that they also impact on rumen metabolism. Q. Wang et al. (2020) demonstrated that ammonia N concentration of rumen fluid increased in dairy cows exposed to AFB_1, ZEA and DON in contaminated diets, suggesting an impact of these mycotoxins on the rumen microbial composition. Next generation sequencing has allowed modern researchers to investigate the interactions of mycotoxins within the rumen microbiome due to high-throughput techniques and the increasing economic feasibility of the analysis (Kulski, 2016). As a result, secondary impacts on the rumen microbiome, of regulated mycotoxins known to be detrimental to ruminant health are continuing to be elucidated (Hartinger et al., 2023).

Grass silages however are commonly found to contain non-regulated mycotoxins, such as PEN (O'Brien, 2010), beauvericin (Manni et al., 2022), MPA (Schneweis et al., 2000), and FUS (Penagos-Tabares et al., 2022), that besides possessing some immunosuppressive activity (e.g. MPA (Bentley, 2000)) can also be antifungal and antimicrobial (Geiger and Conn, 1945; Zhang et al., 2016, 2021). Their lack of severity with regards to immediate hepatoxic, nephrotoxic or carcinogenic effects on animals have meant that their impact on ruminants has largely been uncharacterised. As such there is a paucity of information surrounding the impact of common grass silage mycotoxins on the rumen metabolism and microbiome.

1.4.1. The effect of mycotoxins on rumen metabolism and the microbiome

Previously studies that had investigated interactions between mycotoxins and the rumen microbiome were concerned with demonstrating the ability of rumen microbiota to degrade certain metabolites. In 1966 Ciegler et al. demonstrated the ability of rumen microorganisms to degrade AFB_1 and in 1976 Hult et al. was able to demonstrate rumen ochratoxin A degradation which led to the generally accepted principle that ruminant animals were less sensitive to the exposure of mycotoxins in feed than monogastric animals (Loh et al., 2020). Whilst this may be the case for certain mycotoxins, most of these earlier experiments were carried out in *in-vitro* settings with the inclusion of a singular mycotoxin – not representative of the mycotoxin load an animal may be exposed to when consuming contaminated feed. Furthermore, Westlake et al. (1989) demonstrated that bacterial populations of the ovine rumen were able to degrade the trichothecene HT2-toxin, but that the protozoal fraction were inhibited by exposure, suggesting differential impacts of mycotoxins on certain ruminal groups.

In addition, mycotoxins have been demonstrated to exhibit varied effects when in combination with one another. Grenier and Oswald (2011) classified these interactions into four groups: i) synergistic, ii) additive iii) less than additive, and iv) antagonistic. In addition the same combination of mycotoxins can exhibit both synergistic or antagonistic effects dependent on species and target, resulting in complexity when evaluating the risk of a feed (Speijers and Speijers (2004)).The range of mycotoxin profiles between particular feeds (Jelinek et al., 1989; Rodrigues and Naehrer, 2012) and the numerous interactions therefore with the microbial ecosystem make this area of study challenging, as there are highly varied animal responses to exposure of different combinations (Kruger Ben Shabat et al., 2016).

For example, during *in-vivo* studies to date that have mostly investigated DON, ZEA and FUM, Batista et al. (2024) demonstrated a reduced DM intake when DON at 1423 µg/kg, ZEA at 101 µg/kg and FUM at 4544 µg/kg fed to steers for 29 d, whilst McKay et al. (2019) who investigated DON at 1966 µg/kg DM and ZEA at 366 µg/kg in dairy cows for 29 d did not observe any effect on DM intake. Other studies have shown mixed responses of rumen fermentation with regards to contamination of *Fusarium* mycotoxins, with no effect on acetate, propionate or butyrate, but an effect on valerate (Rivera-Chacon et al., 2024), lowered propionate (Seeling et al., 2005), lowered isovalerate and, increased isobutyrate (Batista et al., 2024) and no effect of contamination on any VFA concentration (Duringer et al., 2020).

Moreover, additional factors regarding the current health status of the animal, diet and genetic composition of the rumen microbiota, all influence heavily on the ability of the ruminant to degrade mycotoxins (Debevere et al., 2020a). The extent of rumen microbial “detoxification”

of certain mycotoxins therefore remains a highly debated area of research (Loh et al., 2020). Nevertheless, the effects of mycotoxin inclusion on ruminant metabolism, microbiome and animal performance, either during *in-vitro* fermentations with rumen fluid or within a diet fed to ruminant animals are described in Table 1-4.

Table 1-4. Animal and *in-vitro* studies that have investigated the effect of mycotoxins on rumen metabolism and the composition of the rumen microbiome.

Animal/study design	Mycotoxin treatment and level of inclusion (where applicable)	Diet provided	Exposure/ feeding duration	Result	Reference
Nellore steers	1) Contaminated diet mainly with FUM 4544 µg/kg DON + TRI 1423 µg/kg ZEA 101 µg/kg 2) Contaminated diet as above with MB	TMR: 120 g/kg DM sugarcane bagasse, 618 g/kg DM corn, 150 g/kg DM citrus pulp, 80 g/kg DM soybean meal, 32 g/kg DM feedlot premix	29 d	Contamination negatively impacted DMI Tended to lower rumen pH No effect on total VFAs Isovalerate proportion was reduced Isobutyrate proportion was increased	Batista et al., (2024)
RUSITEC *In-vitro* using rumen fluid from four sheep	Fusarium contaminated wheat with either DON at 6.9 or 5.8 mg/kg DM in Diet C60 and Diet C30 respectively	Four mixed diets Diet C60 (40 % GS + MS at 50:50, 60 % concentrates) Diet C30 (70 % GS + MS at 50:50, 30 % concentrates)	48 h	Inclusion of contamination decreased concentration of isobutyrate No effect on any other VFAs No effect on rumen ammonia N concentration	Boguhn et al., (2010)
Holstein dairy cows (lactating)	1) TMR including DON < 650 µg/kg ZEA < 107 µg/kg FUM < 280 µg/kg 2) TMR including DON < 1000 µg/kg ZEA < 260 µg/kg FUM < 280 µg/kg 3) TMR as in (2) + MB	TMR: forage (MS, sorghum silage and hay) to concentrate ratio of 49.9:50.1	54 d	Treatment 2 resulted in lower milk yield and feed efficiency	Catellani et al., (2023)

Holstein dairy cows (lactating)	1) DON 0 mg/kg DM concentrate 2) DON 6 mg/kg DM concentrate 3) DON 12 mg/kg DM concentrate	Received 9 kg/d concentrate and forage (70 % MS, 20 % grass and alfalfa silage and 10 % grass hay.	10 w	No effect on milk production No effect on DMI No detectable residues of DON or DOM-1 in milk.	Charmley et al., (1993)
German Holstein dairy cows (mixed lactating and dry)	Wheat contamination with DON and ZEA (DON approx. 8.05 mg kg DM, and ZEA approx. 0.26 mg/kg DM)	50 % wheat, 50 % hay or GS.	4 w	No effect on rumen pH No effect on VFA concentrations Postprandial ammonia concentration was increased Reduced flow of microbial protein at the duodenum	Dänicke et al., (2005)
German Holstein dairy cows (lactating)	1) ZEA 0.02 mg/kg DM DON 0.06 mg/kg DM 2) ZEA 0.29 mg/kg DM DON 2.31 mg/kg DM 3) ZEA 0.58 mg/kg DM DON 4.61 mg/kg DM	TMR: 50 % GS, 50 % concentrates	13 w	Potential effects on regulatory mechanisms involved in water and electrolyte balance	Dänicke et al., (2017)
In-vitro Holstein Friesian dairy cow rumen fluid	1) DON 0 mg/kg 2) DON 5 mg/kg 3) DON 10 mg/kg	2 levels of starch - 20.85% - 27.78 %	24 h	Under low starch, DON addition increased the relative abundance of *Ruminococcus gauvreauii* Under high starch, DON addition increased the relative abundances of *Papillibacter* Under high starch, *Lachnospiraceae AC2044*, *Desulfovibrio* and *Selenomonas* decreased with increasing DON dose.	Dong et al., (2024)

Beef cattle	1) DON 1.7 mg/kg TMR + FUM 3.5 mg/kg TMR	TMR: tall fescue hay, concentrates.	21 d	Decreased ruminal fluid pH No effect of contamination on rumen VFAs	Duringer et al., (2020)
Combined *in-vivo* Holstein dairy cows (lactating) *and in-vitro*	1) DON 341 µg/kg DM FUM 128 µg/kg DM 2) DON 733 µg/kg DM FUM 994 µg/kg DM 3) DON 897 µg/kg DM FUM 1,247 µg/kg DM + 35 g/head/d MB	TMR: 44 % forage including MS	3 x 21 d	Effects on the kinetics of gas production of different feeds	Gallo et al., (2021)
Holstein dairy cow (dry)	ZEA 5 mg/kg DM FUM 20 mg/kg DM	TMR: forage 600 g/kg (20 % GS, 40% MS) grain 400 g/kg	2 d	Reduction in abundance of rumen *Lachnospirachaea* and *Prevotella* FUM increased *Ruminococcaceae* Lower rumen pH and total SCFA concentration	Hartinger et al., (2022)
Holstein dairy cow (dry)	ZEA 0.449 mg/kg DM	Forage rich (50% hay, 50% GS) diet + 2 kg of concentrates	1 d	Increased protozoal abundance from 0-10 h post feeding, returning to normal after 10 h SCFA concentration 4 h post feeding were increased Abundance of *Succinivibrionaceae* UCG-002 increased on day of exposure after feeding Abundance of *Bacteroides pectinophilus* increased after exposure	Hartinger et al., (2023)
In-vitro Holstein rumen fluid	DON 40 mg/kg DM	Corn starch or cellulose	6 h	Reduction in acetate and propionate concentration DON degraded at a higher rate with cellulose diet, compared to corn starch diet	Jeong et al., (2010)

				Reduction in culture ammonia N concentration over time with DON addition	
German Holstein dairy cows (lactating)	1) Control 2) DON 5.3 mg/kg DM then 1) Control 2) DON 4.4 mg/kg DM 3) DON 4.6 mg/kg DM	TMR: 50 % MS and GS, 50 % concentrates for 11 w, followed by Either 30 or 60 % concentrates for 18 w	11 w then 18 w	No effect on milk production or composition as a result of mycotoxin contamination (other effects were observed but as a result of concentrate inclusion).	Keese et al., (2008a)
Holstein Friesian x Red Sindhi	DON 720 µg/kg FB_1 701 µg/kg ZEA 541 µg/kg OTA 501 µg/kg AFB_1 38.0 µg/kg T-2 270 µg/kg (+ a MB)	TMR: Local TMR of ground rice straw and cassava chip		Rumen pH, total VFAs and acetate increased in the contaminated TMR + binder in comparison to just contaminated TMR; propionate decreased respectively. Bacterial and protozoal counts lower in the rumen of cows fed contaminated TMR + binder in comparison to just contaminated TMR	Kiyothong et al., (2012)
Holstein dairy cows (lactating)	Contaminated TMR (DON was the major contaminant found at 3.5 mg/kg DM)	TMR: including MS and hay.	63 d	No effect on DMI No effect on milk production or milk composition Treatment increased serum sodium concentrations. No effect on rumen fluid ammonia concentration Neutrophil phagocytosis decreased in treatment fed cows.	Korosteleva et al., (2009)
In-vitro culture of *R. albus* + *M. ruminantium*	FUS at either 15, 30, 45, 60, 120, 240, 480 µg/mL DON at 100 µg/mL PIC at 41.5, 83.1 or 369.3 µg/mL	N/A. Provided nutrition for culture in the medium	0-28 d	Growth of *Ruminococcus albus* and *Methanobrevibacter ruminantium* were inhibited by FUS from 15 µg/mL, but not DON.	May et al., (2000)

				At 480 µg/mL FUS, *M. ruminantium* did not grow, *R. albus* grew slowly for one week.	
Holstein Friesian dairy cows (lactating)	1) DON 163 µg/kg DM ZEA 19.0 µg/kg DM 2) DON 1,966 µg/kg DM ZEA 366 µg/kg DM	TMR: 61 % forage (MS and GS), 39 % concentrates	28 d	No effect of treatment on DMI, milk production or composition. Treatment 2 resulted in a 0.74 kg/cow/d decline in milk yield.	McKay et al., (2019)
Simmental cows (lactating)	ZEA 9.45 mg/d	TMR: forage 600 g/kg (20 % GS, 40% MS) grain 400 g/kg	21 d	Acetate, propionate and butyrate concentrations did not change following exposure Valerate concentration decreased with increasing duration of exposure	Rivera-Chacon et al., (2024)
RUSITEC *In-vitro* with fluid from 4 Holstein dairy cows (lactating)	1) DIET + 1 g/d MB 2) DIET + 20 mg/kg EA 3) DIET + 20 mg/kg EA + 1 g/d MB	DIET: 84.8 % barley grain, 10 % barley silage, 5.2 % minerals and vitamins	14 d (including 7 d adaptation period)	EA reduced DM disappearance. EA decreased acetate proportion EA increased isovalerate proportion EA reduced acetate:propionate No effect of ammonia, total gas, pH or methane EA reduced *Verrucomicrobiota* No effect of EA on general abundance of *firmicutes*, *bacteroidota* or *proteobacteria*.	Sarich et al., (2021)
German Friesian dairy cow (mixed lactating and dry)	DON 8.21 mg/kg DM ZEA 0.09 mg/kg DM	TMR: 40% forage (50 % GS, 50 % MS) 60 % concentrates (55 % either contaminated wheat or control)	4 w	Lower propionate concentration with treatment No effect on rumen pH No effect on milk yield, milk fat or protein	Seeling et al., 2005

Holstein Friesian dairy cows (lactating)	1) 25 % silage replacement with spoilt silage 2) 25 % silage replacement with spoilt silage + MB	TMR: 69 % GS, 31 % concentrates	5 d	DMI unaffected by treatment Milk fat and protein content unaffected by treatment Rumen metabolism was unaffected by treatment (pH and VFAs)	Snelling et al., (2021)
Holstein dairy cows (lactating)	1) Diet + AFB 404 µg, DON 5025 µg, FUM 8046 µg, T2-toxin 195 µg, ZEA 2008 µg. 3) as above + MB1 4) as above+ MB2 5) as above + MB3	TMR diet: 48 % MS, 52 % concentrates	7 d	No effect on DMI No effect on milk yield or milk composition	Vieira et al., (2024)
Holstein dairy cow (late lactation)	AFB_1 20.08 µg/kg DM DON – not detected ZEA 85.13 µg/kg DM	TMR: MS with cottonseed, either 50%, or 100% replacement with contamination of all three mycotoxins		Rumen ammonia N concentrations greater in 100% replacement No level of replacement affected the total VFAs or individual concentrations, except isovalerate.	Q. Wang et al., (2020)
Holstein dairy cow (lactating)	1) ZEA 0.02 mg/kg DM DON 0.07 mg/kg DM 2) ZEA 0.33 mg/kg DM DON 2.62 mg/kg DM 3) ZEA 0.66 mg/kg DM DON 5.24 mg/kg DM	TMR: 50 % GS, 50% concentrates	13 w	No effect of treatment on body weight, DM intake or energy balance. No effect of treatment on milk production or composition	Winkler et al., (2014)

MB = mycotoxin binder, FUM = fumonisins, FB_1 = fumonisin B_1, ZEA = zearalenone, DON = deoxynivalenol, EA = ergot alkaloids, OTA = ochratoxin A, AFB_1 = aflatoxin B_1, T-2 = T-2 toxin, PIC = picolinic acid, FUS = fusaric acid, TRI = trichothecenes, GS = grass silage, MS = maize silage, h = hour(s), d = day(s), w = week(s),TMR = total mixed ration, DM = dry matter, DMI = dry matter intake, VFAs = volatile fatty acids

1.5. Mitigation with mycotoxin binders and adsorption agents

A range of mycotoxin binders and adsorption agents are available commercially and are listed in Table 1-5. Some products can be added to the forage before ensiling or added directly in to the TMR at feeding and are mainly composed of silicate clays such as bentonite and smectite (Zabiulla et al., 2021), yeast *(Saccharomyces cerevisiae)* cell walls (Yiannikouris et al., 2005), and enzymes (Debevere et al., 2020b). They can be classified into two groups, binders or modifiers, where clays and yeast cell walls comprise the former, and enzymes or bacteria comprise the latter (Kihal et al., 2022). Binders render the mycotoxin inert, preventing absorption by the animal, whereas modifiers carry out biotransformation of the mycotoxin into less hazardous or harmless metabolites (Kihal et al., 2022). The formulation of mycotoxin binders has become more sophisticated over recent years, including the development of effective broad-range binders that no longer interfere with the bioavailability of the mineral content of feed, and have been reviewed extensively by Whitlow (2006), De Mil et al. (2015) and Kihal et al. (2022).

Overall, the first point of defence against livestock exposure to mycotoxins should aim to inhibit the synthesis of mycotoxins in feed for livestock, rather than a reliance on mitigation strategies, as it may not be immediately obvious that an animal is experiencing mycotoxin exposure, due to the mixed species responses to mycotoxins (Bertero et al., 2018). Furthermore, Kolawole et al. (2019) reported that only 1 out of 10 commercial mycotoxin mitigation products tested was able to adsorb more than 50 % of DON, ZEA, fumonisin B_1, ochratoxin A and AFB_1, with the yeast cell wall binder being most effective. Additionally, factors such as mycotoxin profile of a feed, and the conditions within a specific animal's digestive system, could interfere with the efficacy of mycotoxin binders and modifiers (Kolawole et al., 2019).

Table 1-5. A selection of mycotoxin binding products available commercially in the UK (Product names and formulations are correct as of August 2024). Not all available products are listed.

Product Name	Company	Description stated by manufacturer	Ingredients
Anpro®Advance	Anpario®	Broad-range and aflatoxin B_1	Silicate clays, diatomite
Calibrin®Z	Amlan Int.	Broad-range	Silicate clays
Elitox®	Impextraco®	Broad-range	Chitosan and silicate clay
Escent®	Innovad®	Broad-range	Yeast, silicate clay, organic acids
FUMzyme®Silage	DSM®	Fumonisins (silage)	Minerals, FUMzyme®, and algae
Kallsil®	Kemin®	Broad-range	Silicate clays
Mastersorb®	EW Nutrition®	Broad-range (+ endotoxins)	Yeast and silicate clay
MT.X+®	Olmix®	Broad-range	Silicate clays and algae
Mycofix® Plus	DSM®	Broad-range	Minerals, enzymes, and algae
Mycofix®Plus 5.Z	DSM®	Broad-range + zearalenone	Minerals, ZENzyme®, and algae
Mycosorb®A+	Alltech®	Broad-range	Yeast and algae
Notox™	Cargill®	Fumonisins and aflatoxins	Silicate clays
Novasil™ Plus	BASF®	Aflatoxins	Silicate clays
TOXO®	Selko®	Aflatoxins and ergot alkaloids	Silicate clays
Toxy-Nil®	Adisseo	Broad-range	Yeast, silicate clay
UltraSorb R	Volac®	Broad-range and deoxynivalenol	Bacteria, yeast and silicate clay
Vitafix®	Agrimprove	Broad-range (+ endotoxins)	Silicate clays
X-Bond®	Vilomix®	Broad-range	Yeast, enzymes and minerals

1.6. Conclusions from the literature

- Fungi are ubiquitous within the environment and so attempts to eliminate fungal contamination from feed are not feasible
- Grass silages are a predominant component of dairy feed in Northern Europe
- Methods of silage management have been generally suggested to reduce mycotoxin production, but the triggers for mycotoxin synthesis by fungi in grass silage are not well understood
- Furthermore, where mycotoxin effects on cattle have been studied, maize silages have often been the focus rather than grass silages due to the higher incidence of regulated mycotoxins in maize
- Grass silages often contain non-regulated mycotoxins that can possess immunosuppressive and antibacterial properties, that may still disrupt rumen fermentation
- Some rumen microorganisms can degrade certain mycotoxins, but not all
- Furthermore, under illness or metabolic stress, the ability of rumen microorganisms to degrade mycotoxins may be compromised
- There exists little information on the effect of mycotoxins on the rumen microbiome
- There may be antagonistic or synergistic interactions between mycotoxins on the rumen microbiome
- Grass silage mycotoxins currently pose an unknown risk to dairy cow performance

1.7. Main objectives for study and thesis outline

i) Investigate the presence of grass silage mycotoxins on farms in the United Kingdom and establish the mycotoxin profile of these grass silages.
ii) Investigate any relationships between the presence of mycotoxins and the production methods, clamp management methods and fermentation profile of grass silages in the United Kingdom.
iii) Investigate the effect of grass silage mycotoxins on the rumen metabolism *in-vitro*
iv) Investigate the effect of grass silage mycotoxins on the rumen metabolism, the rumen microbiome and subsequent performance of dairy cows *in-vivo*.

Please note that the experiments detailed in this thesis were carried out in a different order to that in which they are presented.

Areas that are highlighted on the map in blue will be discussed in detail in this thesis (Figure 1-14). Connectors display the links between study areas relating to mycotoxins. Though not all areas below will be explored in this thesis, this map provides a visual aid to understanding how the work discussed fits within the wider context of this research area.

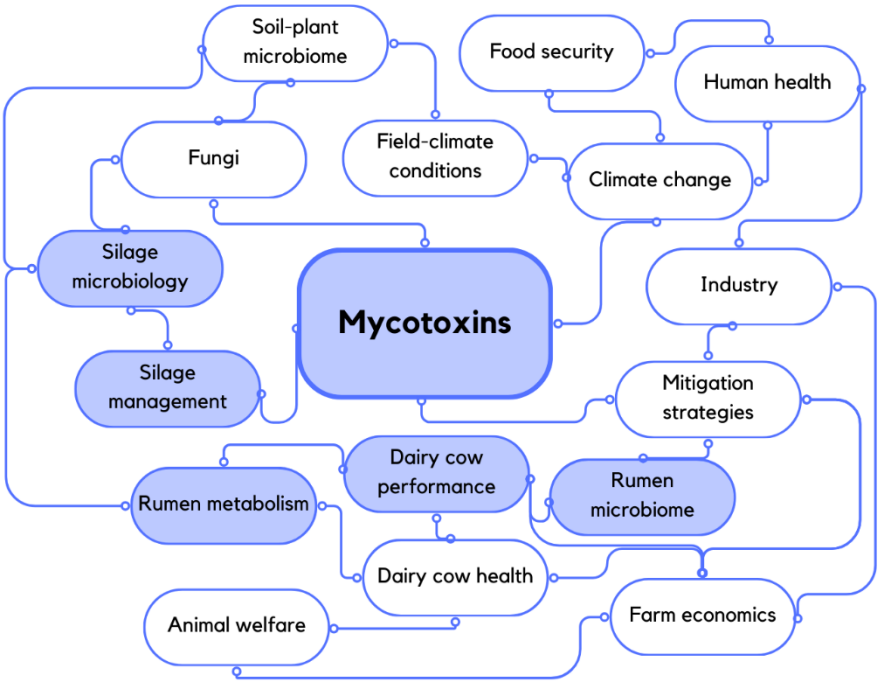


Figure 1-14. Thesis map detailing the links between topic areas. Topics highlighted in blue are those that will be explored and discussed in detail in this Thesis.

Chapter 2

General materials and methods

2. General materials and methods

2.1. Determination of rumen VFA concentration

Rumen fluid concentrations of acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate were quantified based on the method by Erwin et al., (1961) via gas chromatography (GC; Agilent 8860 GC System, Agilent Technologies™, California, United States) and using DB-FFAP column, model 122-3232 with a film thickness of 0.25 µm, length of 30.0 m and diameter of 250.0 µm (Agilent Technologies™, California, United States). The parameters of the GC were: front inlet temperature: 250°C, temperature range: 40°C – 250°C, pressure: 11.627 psi, flow: 2 ml/min, heater: 300°C, split ratio: 20:1, split flow: 40 ml/min and total flow: 42 mL/min.

Strained rumen fluid samples were collected into 50 ml falcon tubes (45 ml) prepared with 5 ml of 25% w/v metaphosphoric acid (Sigma-Aldrich® , Dorset, United Kingdom) and frozen at -20°C. Samples were thawed, thoroughly vortexed and 20 ml was dispensed into 50 ml Nalgene high-speed centrifuge tubes (Thermo Fisher Scientific, Massachusetts, United States), and spun at 16000 xg for 20 minutes at 4°C in a benchtop refrigerated centrifuge (Sigma, Osterode, Germany). Using a pipette, 15 ml of the supernatant was then transferred into a clean 15 ml falcon tube ready for GC vial setup. For the *in-vitro* experiments, a 1 ml sample of each *in-vitro* culture was pipetted straight into 1.5 ml Eppendorf tubes and topped up with 200 µl of 25% w/v metaphosphoric acid, before incubating in the freezer overnight. The following day, the Eppendorf tubes were thawed, vortexed thoroughly and 1 ml of the supernatant was transferred into clean 1.5 ml Eppendorf tube.

A 1 ml sample of the supernatant from each sample was transferred into 2 ml clear robotic GC vials, with screw cap and PTFE septa, (Thermo Fisher Scientific, Massachusetts, United States) in duplicate. Vials containing 2 ml of HPLC grade chromatography water (Thermo Fisher Scientific, Massachusetts, United States) were prepared with a water vial placed between every 10 samples to provide additional cleansing alongside a pre-programmed clean with oxalic acid (5 % v/v). Using a pipette, 100 µl of internal standard (ethyl-butyric acid) was added to each sample vial. An external standard of 1 ml volume was also added to a GC vial, along with 100 µl of the internal standard. The external and internal standards were prepared as detailed in Table 2-1.

Table 2-1. Components of the external and internal standards used in the determination of volatile fatty acids by gas chromatography.

VFA standard	Volume (µl)	FW (g/mol)	Density (g/ml)	Molarity (mM)
External standard				
Acetic acid	172	60.05	1.049	30.0
Propionic acid	149	74.08	0.993	20.0
Isobutyric acid	47.0	88.11	0.950	5.00
Butyric acid	184	88.11	0.964	20.0
Isovaleric acid	55.0	102.1	0.930	5.00
Valeric acid	54.0	102.1	0.939	5.00
Internal standard				
2-ethyl butyric acid	1.263 ml	116.16	0.920	100

Both external and internal standards made up to 100 ml with HPLC grade H_2O.

Identification of each VFA was determined according to retention time of pure standards. Quantification was calculated as the area of the peak at the specific retention time for each VFA (Table 2-2), compared with the standard curves determined from the external standard.

Table 2-2. Peaks of each volatile fatty acid (VFA) and the retention time within the gas chromatograph column.

VFA peaks	Retention time (minutes)
Acetic acid	4.3
Propionic acid	4.8
Isobutyric acid	5.0
Butyric acid	5.4
Isovaleric acid	5.6
Valeric acid	6.1
2-ethyl butyric acid	6.2

2.2. Rumen ammonia N concentration

Rumen samples that had been spun down at 16000 xg for 20 minutes at 4°C in preparation for the VFA analysis were used for the ammonia N determination by steam distillation. A 5 ml sample of supernatant was added to a boiling tube with 6 ml of 96% magnesium oxide (Thermo Fisher Scientific, Massachusetts, United States), and placed into a steam distillation unit (FOSS Kjeltec™ 8100, FOSS, Cheshire, United Kingdom). Sample analysis was carried out in duplicate. The acid used in the titration was 0.005 M sulfuric acid (Thermo Fisher Scientific,

Massachusetts, United States), and the calibration was carried out to 100% (± 2.00%) recovery with 5 ml of an ammonium standard (1000 mg ammonium chloride/L in water; VWR, Pennsylvania, United States) with 6 ml of 96% magnesium oxide. The equation for the determination of ammonia N is provided in Equation 8.

Equation 8. Determination of ammonia N concentration of rumen fluid samples by the Kjeldahl method

$$\text{Ammonia N (mg/L)} = \frac{\text{mean titre value - blank value}}{0.005} \times 0.14$$

2.3. Chemical analyses of forage and total mixed ration (TMR) samples

2.3.1. Dry matter determination

DM determination was carried out on all forage and TMR samples by drying in an oven at 60 ºC under <100 mm Hg, to constant weight, following Equation 9, as described in AOAC method 934.01 (AOAC, 2012).

Equation 9. Determination of dry matter in forage and TMR samples

$$\text{Dry matter g/kg} = \frac{\text{weight of sample before drying}}{\text{weight of sample after drying}} \times 1000$$

2.3.2. Crude protein

The crude protein of forage or a TMR was carried out according to the Dumas method (AOAC, 2012; 988.05). Samples were dried in an oven at 60 ºC to constant weight and then milled using a hammer mill through a 1 mm screen. Nitrogen determination was carried out via combustion followed by thermal conductivity cell detection of nitrogen gas (N_2) on a LECO CN 828 (LECO, Michigan, United States). Calibration was carried out using 0.15 g of EDTA. Crude protein was determined from the total nitrogen of the sample using Equation 10:

Equation 10. Calculation of forage crude protein (g/kg DM)

$$\text{Crude protein (g/kg DM)} = \text{total nitrogen (g/kg DM)} \times 6.25$$

2.3.3. Neutral detergent fibre and ash

Neutral detergent fibre (NDF) was determined according to the method outlined by Van Soest et al. (1991). Approximately 0.5 g of dried and milled forage was weighed into a crucible and placed into a Fibretec unit (FOSS Fibretec 1020, FOSS, Cheshire, United Kingdom). A volume

of 25 ml of neutral detergent fibre reagent and 0.5 ml of octan-1-ol were added to each crucible before boiling for 30 minutes. Following boiling, 25 ml of neutral detergent fibre reagent, 2 ml of α-amylase and 0.5 g of sodium sulfite were added to crucibles, before an additional 30 minutes of boiling. Crucibles were then filtered with 25 ml of distilled water at 80°C, before a second addition of 2 ml α-amylase, and 25 ml of distilled water at 80°C, and left for 15 minutes. Crucibles were then filtered again with distilled water and dried at 105°C for at least 18 hours, before cooling in a desiccator and the difference in weight recorded. Total ash was determined according to AOAC (2012) 942.05 by ashing in a muffle furnace at 600 ºC for 2 h. Crucibles were then cooled in a desiccator before weighing again. Neutral detergent fibre was calculated using Equation 11, and expressed exclusive of ash.

Equation 11. Calculation of forage neutral detergent fibre (g/kg DM)

$$\text{Neutral detergent fibre (g/kg DM)} = \frac{\text{Dried weight (g) - Ashed weight (g)}}{\text{Initial sample weight (g)}} \times 1000$$

2.3.4. pH determination of forage

A forage subsample of 10 g fresh weight was placed into a stomacher bag (Seward with 90 ml of deionised water and sealed with a clip top seal (Seward, West Sussex, United Kingdom). Stomacher bags were then loaded onto the stomacher (400 Circulator Seward, West Sussex, United Kingdom) for 2 minutes at 230 rpm before the pH was recorded by submersion of a probe into the supernatant, connected to a benchtop monitor (Jenway 3510, Antylia Scientific, Illinois, United States). Probes were calibrated using pH 4 and pH 7 standards before use.

2.3.5. Forage near-infrared spectroscopy (NIRS) analysis

Dry matter content (g/kg) corrected for loss of volatiles, percentage of lactic acid, acetic acid, crude protein, crude fibre, ash, ammonia N as a % of total N and total VFAs of forage were predicted by near-infrared spectroscopy based on equations developed by the UK Forage Analytical Assurance Group (https://www.faagroup.co.uk/) at Trouw Nutrition (Trouw Nutrition, Derbyshire, United Kingdom).

2.3.6. Forage and TMR mycotoxin analysis

Mycotoxin analysis was carried out by Alltech© 37+ Biotechnology Centre, Dunboyne, Republic of Ireland, following the methods outlined by Jackson et al., (2012). As of 2024, a total of 42 mycotoxins were able to be quantified via this method.

Approximately 250 g fresh weight of samples received were freeze dried for approximately 24 hours until constant weight, before being milled through a 1 mm screen to obtain a homogeneous dried sample. A subsample of 400 mg (± 8 mg) of sample was the weighed in triplicate into individual 20 ml capacity glass test tubes. To each glass tube, 1.6 ml of extraction solution (Table 2-3) was dispensed.

Table 2-3. Components of reagents used in preparation of a forage sample for mycotoxin analysis.

Reagents	In 1L
Extraction solution	
Acetonitrile	840 ml
Deionised H_2O	159 ml
Formic acid	1.00 ml
Loading buffer	
Ammonium acetate (10 mM)	950 ml
Acetonitrile	49.0 ml
Formic acid	1.00 ml

Following the extraction buffer, 20 µl of an internal standard containing known concentrations of ^{13}C isotopic deoxynivalenol, aflatoxin B_1 and zearalenone, together in acetonitrile was dispensed into each tube. A 20 µl volume of known concentration of fumonisin B_1 in a 50:50 mixture of acetonitrile to deionised H_2O, was also added to each tube. Sample tubes were then secured into racks on a rotary laboratory shaker at 250 rpm for a minimum of 8 hours but no longer than 18 hours.

After shaking, 400 µl of the supernatant was transferred to 2 ml chromatography vials with butyl rubber membrane screw caps and centrifuged for 10 minutes at 12,000 rpm (9660 xg) using a laboratory benchtop centrifuge. Samples were then dried under nitrogen at 35 ºC for 1 hour. Samples were then re-eluted with 500 µl loading buffer (Table 2-3) and placed onto an autosampler attached to an ultra-performance liquid chromatography and combined electrospray ionisation tandem mass-spectrometer (Figure 2-1; Acquity UPLC H-Class Plus, and Waters Stepwave ZSpray ESI Mass Spectrometer, Waters Corporation, Massachusetts, United States). The autosampler injected 3 µl of sample provided into the UPLC that was fitted with a C18 2.7 µm 2.1 x 100mm column in triplicate, where it passed through the electrospray ionisation tandem mass-spectrometry unit (ESI-MS/MS) as in Figure 2-2. Conditions for the UPLC and ESI-MS/MS and calibration curves were as described by Jackson et al., (2012).

Mycotoxins were identified via retention time and the quantity was determined via a comparison of the signal to the internal standard.

Figure 2-1. The autosampling unit (left), ultra-performance liquid chromatography unit (centre) and the electrospray ionisation tandem mass spectrometry unit (right).

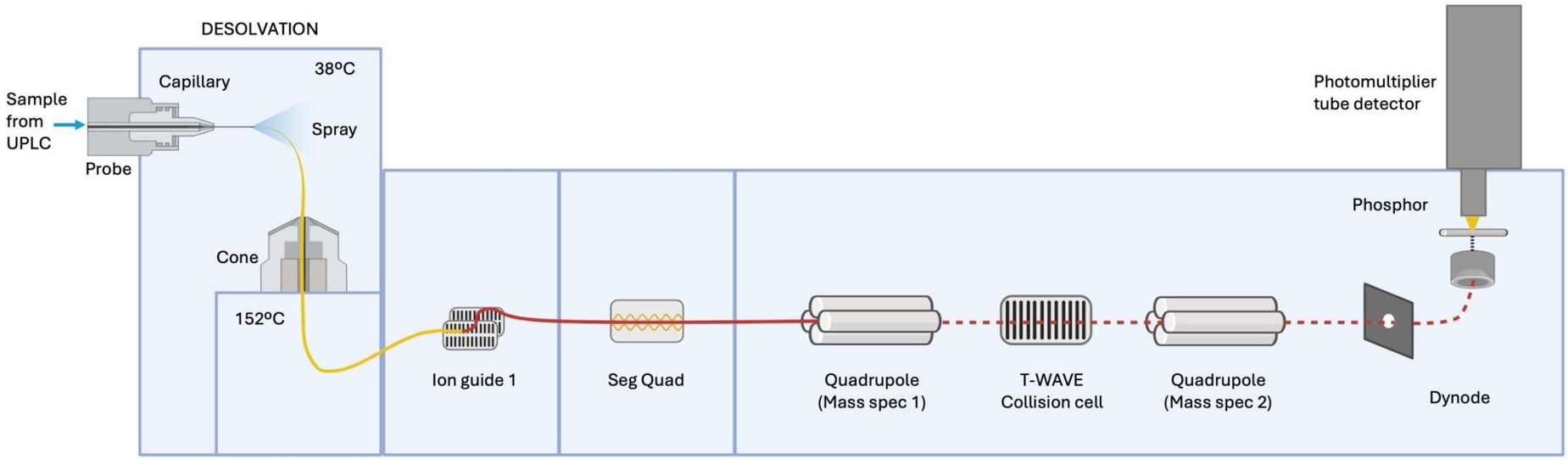


Figure 2-2. Schematic diagram of the electrospray ionisation tandem mass spectrometry system. Sample enters the desolvation phase from the UPLC where it is then accelerated through the cone before passing through a series of ionisation phases, and through the quadrupoles before reaching the photomultiplier tube detector where the mass of the daughter and parent ions are determined. Adapted from display provided by Waters LC-MS/MS software (Waters Corporation, Massachusetts, United States).

2.3.6.1. Determination of the Alltech© risk equivalent quantity

The Alltech© risk equivalent quantity (REQ) was determined from an internal database including results from samples of feed received by Alltech© and informed by the current literature, to provide a toxin equivalent quotient for a particular mycotoxin with respect to aflatoxin B_1. A value is assigned for groups of mycotoxins with their respective effect on a particular species in relative comparison to the toxicity of AFB_1 whilst also taking into account additive and synergistic effects where information is available. The resultant value is a summate that can be used to assess the risk of a feed upon exposure to a certain animal. For mature dairy cows receiving a TMR, REQ values of 0-50 µg/kg DM are considered lower risk, around 100 µg/kg DM are considered moderate risk, and values above that of 150 µg/kg DM are considered higher risk for adverse mycotoxin impacts on animal performance and health.

Chapter 3

The prevalence of mycotoxins in British grass silage and on-farm factors influencing their synthesis: A Survey.

3. The prevalence of mycotoxins in British grass silage and on-farm factors influencing their synthesis: A Survey.

3.1. Introduction

Mycotoxins are secondary metabolites produced by fungi that can elicit mutagenic and immunosuppressive effects on humans and animals (Hussein and Brasel, 2001; Malekinejad and Fink-Gremmels, 2020). Due to the ubiquitous nature of filamentous fungi and plants, production of forages that are free of fungal contamination is not feasible (Pusztahelyi et al., 2015). Understanding the effects of mycotoxins on individual animals is therefore key in developing mitigation strategies to reduce their negative impact on the agricultural industry, as well as animal and human health (Ogunade et al., 2018). The presence of mycotoxins in grass silages are documented in the literature, however information on the factors responsible for inhibiting or promoting mycotoxin synthesis are not fully understood, particularly with regards to the ensiling process (O'Brien, 2010).

Firstly, it is well established that prolonged oxygen exposure of silages, heavily negates the efficacy of the fermentation process required to form a silage of high nutritional quality (Borreani et al., 2018; Orosz et al., 2013). Naturally present lactic acid bacteria that can synthesise lactic and acetic acids required to inhibit undesirable bacterial and fungal growth, are themselves inhibited by oxygen exposure, and so an acidic pH is either not achieved rapidly enough to prevent spoilage occurring, or not achieved at all (Pahlow et al., 2015). Additionally, yeasts and other undesirable organisms can proliferate, compete with lactic acid bacteria for available sugars and raise the temperature within the immediate silage environment, severely impacting on available amino acid content of forages, due to proteolysis (Borreani et al., 2018; Elferink et al., 2000).

"Best-practice" advice for silage-making would therefore first and foremost include that a highly anaerobic environment must be maintained throughout the duration of the fermentation and into the stable phase (Pahlow et al., 2015). Compaction of the grass throughout the filling stage and a uniformly weighed down air-tight covering securing the clamp once filling has finished, would ensure that oxygen would be quickly limited within the silage environment, as plant tissues continue to respire (Borreani et al., 2018). Silage additives of chemicals or inoculants of obligate homofermentative lactic acid bacteria (LAB), facultative heterofermentative LAB or obligate heterofermentative LAB, or a mixture of all, can be applied to ensure rapid acidification of the silage environment (Soundharrajan et al., 2021). Once a successful fermentation is achieved, silage can remain in this stable state of preservation until required for feeding (Elferink et al., 2000).

However, exposure of the ensiled clamp to oxygen occurs again upon opening for feeding and so the preserved forage is again at risk of spoilage (Davies et al., 2018). Upon opening, a race begins to feed out silage more rapidly than it can suffer nutritional losses through oxygen exposure to the face of the clamp, in particular (Bernardes et al., 2021). Therefore, good management of silage upon opening and subsequent feed out, is as critical at this time, as at the point of ensiling.

Irrespective of following the above guidelines, filamentous fungi and fungal spores are still ubiquitous within silages and their harmful products, mycotoxins, have been found in visibly non-moulded silage (Yoshihara and Miyazaki, 2023). This not only means that the mitigation process for mycotoxin proliferation may be more challenging than simply implementing practices that promote effective forage preservation, but also poses a significant risk to livestock, as there could be a lack of accompanying visual or olfactory warnings that a silage may be hazardous. Mycotoxin analysis of feed is offered by many companies, from small test kits with rapid results for a limited range of mycotoxins, to more comprehensive laboratory tests using a combination of liquid chromatography (LC) and mass spectrometry (MS) techniques (Jackson et al., 2012; Maggira et al., 2022). Even though the latter benefits from more reliable results and the ability to quantify a greater range of metabolites, all tests are performed on already produced feed, and so by this stage, there are no opportunities for prevention, only remediation of existing mycotoxin levels. A study by Mansfield et al., (2005) aimed to establish correlations between agronomic practices, fermentation characteristics and DON in ensiled maize, but could not conclude any such relationships. As this study was carried out with respect to a single mycotoxin only and in maize silages, almost twenty years ago, it would be inappropriate to apply the findings to the situation of grass silage in Great Britain today. As of the present there have been no relationships established between grass ensiling methods employed on-farm and their resulting mycotoxin profiles.

To exacerbate the issue, weather conditions brought about by climate change, are only predicted to favour the growth of fungi in temperate climates in the future (Kos et al., 2023), and so the profile of prominent grass silage mycotoxins may change over time. Furthermore, the exact methods of harvesting and ensiling can greatly differ between farms due to cultural, economic and educational factors (Barnes et al., 2019; Tiffin and Balcombe, 2011; Vanclay and Enticott, 2011) which may pose a challenge to tackling mycotoxin contamination. It is therefore important to identify any relationships between current farm management strategies across Great Britain, silage chemistry and their associated mycotoxin profiles, in order to offer tailored advice to individual farms. There has already been a general shift towards accounting for differences between farming practices when calculating metrics such as carbon footprints,

with region-specific or practice-specific recommendations for improvement (Sorley et al., 2024). It is not inconceivable therefore, to imagine a future service offering recommendations specifically curated for each farm for their most effective grass silage mycotoxin preventative strategy for that year's harvest and ensiling.

A tailored service to critically evaluate the loss of silages to waste is already currently offered by Alltech© UK., Ltd. The service, *Navigate*™, developed by Dr. David R. Davies of Silage Solutions Ltd., in partnership with Alltech© UK Ltd., subjects farms to an audit where on-farm measurements are combined with silage fermentation profile data, obtained by near-infrared spectroscopy (NIRS), to offer recommendations for more efficient silage production and management; reducing financial losses from wastage. In this study, building upon the existing *Navigate*™ template, silage mycotoxin analysis and wet chemical analysis of the silages sampled were also included in order to increase the breadth of silage fermentation data captured at each farm.

3.2. Objectives

By combining data obtained through the modified *Navigate*™ survey, fermentation and mycotoxin profiles and mycotoxin concentrations within the grass silage sampled, this study aimed to achieve the following objectives:

1) Understand the typical mycotoxin profile of grass silages in Great Britain
2) Elucidate any potential relationships between mycotoxin profile and concentrations, and grass silage nutritive value, fermentation profile, and the current farming practices employed on dairy farms in Great Britain.
3) Provide information for the development of more effective mycotoxin mitigation strategies on farm.

3.3. Hypotheses

Following the current 'best-practice" advice provided in the literature regarding harvesting methods and silage clamp management-based mycotoxin mitigation strategies, the following main hypotheses were established for this study.

1) grass silage that had achieved a "successful" fermentation, assessed by the wet chemistry of the silage and the management methods utilised, would display a negative relationship with concentrations of all grass silage mycotoxins.
2) grass silage that had achieved a "successful" fermentation, assessed by wet chemistry of the silage, but then experienced poor clamp management upon reopening, would

display a positive relationship with concentrations of grass silage mycotoxins, in particular, those associated with aerobic spoilage.

3) conversely, grass silage that had not achieved a “successful” fermentation, assessed by wet chemistry of the silage would display a strong positive relationship with concentrations of all grass silage mycotoxins.

3.4. Materials and methods

3.4.1. Selected farms for the study

Farm visits took place during March and April 2022, and again during March 2023. A total of thirty-six dairy farms (thirty-seven grass silage clamps) were identified in the South West of England (19), Wales (3), the West Midlands (5), the East Midlands (2) and Southern Scotland (8; Figure 3-1). Farms were selected based on the criteria that they were, at the time of visit, working commercial dairy farms in Great Britain, involved in the production and management of grass silage on-farm and currently fed to their dairy herd. All farms were customers of Alltech® (Alltech© UK Ltd., Stamford, United Kingdom) or TBAgri (Trevor Birchall Agriculture Ltd., Dorset, United Kingdom). Farms with a known previous mycotoxin contamination history and farms without, were included in this study. All farms provided consent to be involved in the study prior to the visit.

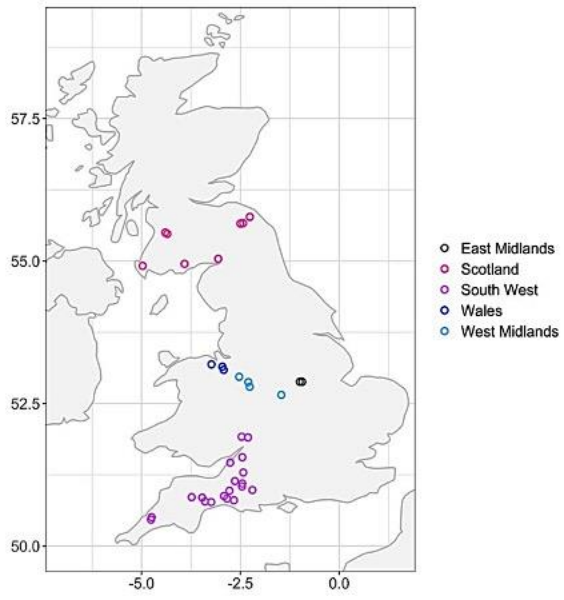


Figure 3-1. A total of thirty-six dairy farms (thirty-seven grass silage clamps) were visited over March – April 2022 and again during March 2023 for this study including regions of

Southern Scotland, Northern Wales, the West Midlands, the East Midlands and the South West of England.

3.4.2. Clamp dimensions and questionnaire

Length, width and height of each clamp was recorded using a handheld measuring wheel (Screwfix, Yeovil, United Kingdom). Any visible mould, visible soil contamination across the clamp face and presence of effluent, were also recorded. Landscape photographs of the clamp face were taken using a mobile phone (Figure 3-2).

Figure 3-2. A photograph of an earth-banked grass silage clamp that was sampled from in the study.

A survey of twenty-four questions relating to conditions at harvest, methods of ensiling and management of the clamp were developed based upon the current best-practice recommendations for farms during an Alltech© Navigate™ Survey, as directed by Dr. David Davies of Silage Solutions Ltd. (Silage Solutions Ltd., Wales, United Kingdom). Questions were designed to elucidate whether a silage fermentation had experienced any factors that could lead to the proliferation of spoilage organisms as highlighted in the literature. For example, regarding the weather conditions at harvest, use of organic or inorganic fertilisers, silage additive used, density of compaction, and maintenance of anaerobicity throughout ensiling and also at feed-out. All farmers provided written consent before participation in the study. Where farmers were not present during the visit, questions were asked over telephone call.

3.4.3. Sampling silage from the clamps

Grass silage samples were obtained from cores taken at a depth of ~15 cm drilled horizontally into the clamp face, from the “top” and “mid” sections, as shown in Figure 3-3.”Top” cores were defined as approximately 15 cm lower than the top height of the clamp, with one core taken from the centre, and one at each shoulder (left and right) 15 cm inwards from the left or right hand sidewall, respectively. Cores were obtained with a 5 cm dia stainless steel corer (Master forage probe, Dairy One, New York) attached to a petrol drill (STIHL BT 45, STIHL, Surrey, United Kingdom) at ~15 cm depth into the clamp face yielding a sample of ~ 350 g fresh weight (FW) per core. Approximately 300 g FW each of the three “top” cores were bulked together and mixed thoroughly to form one combined “top” sample of ~900 g FW.

“Mid” section samples were obtained with cores taken at approximately 1.5 m high from the ground vertically and drilled approximately 15 cm horizontally into the clamp face. Where the full height of the clamp did not surpass 1.5 m, “mid” sections were taken from an estimated halfway point between the base and the top height of the clamp, ensuring at least a 30 cm vertical distance below the top centre core.

An additional core was obtained from the mid location at the same depth as the first, and bulked in equal fresh weight, to form one combined “mid” sample of ~700 g FW. Triplicate subsamples of ~200 g each from the combined top sample and the mid sample were sealed in zip-lock plastic bags and sent immediately for near-infrared spectroscopy prediction analysis (NIRS, Trouw Nutrition, Derbyshire, United Kingdom), mycotoxin analysis by liquid chromatography – tandem mass spectrometry (LC/MS-MS, Alltech© 37+, Dunboyne, Republic of Ireland) and the third subsample was archived at -20°C before subsequent wet chemical analysis of lactic acid concentration, ammonia N concentration, crude protein and VFA profile (Sciantec, York, United Kingdom).

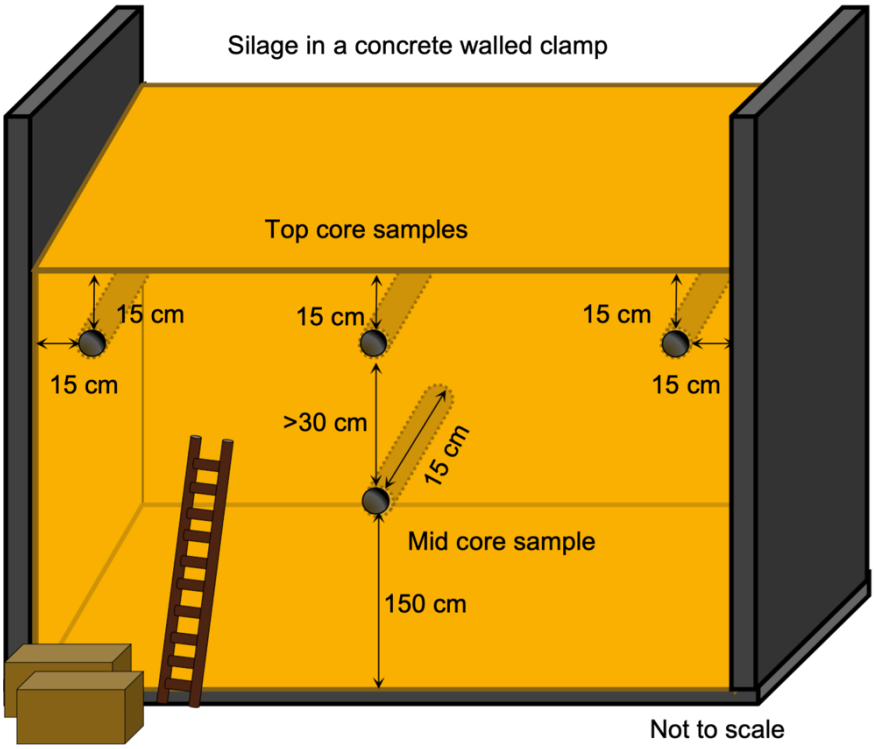


Figure 3-3. Location of the “top” and “mid” core sites drilled across the grass silage clamp faces of the thirty-seven clamps visited during the study.

At each of the four core sites, temperatures at a depth of 50 cm and 10 cm horizontally into the clamp face were recorded with a 50 cm T-shaped stainless steel penetrative temperature probe (ETI, Sussex, United Kingdom), and a 10 cm handheld penetrative temperature probe (Testo instruments, Montreal, Canada), respectively.

3.4.4. Analyses of the data collected

3.4.4.1. Mycotoxin assignment

Mycotoxins were assigned to the following fungal genera *Fusarium, Aspergillus, Penicillium* and *Claviceps* based upon evidence in the literature of the ability of species within each genus to synthesise the specific mycotoxin. Further details of specific mycotoxin assignment are detailed in Appendix Table 1. The concentration (µg/kg based on 88% DM) values for each of the mycotoxins were summed to provide a “Total X Mycotoxins (µg/kg)” value, where “X” represents one of the four fungal genera, as described previously.

3.4.4.2. Statistical analyses

Summary statistics were calculated for clamp dimensions, clamp core densities and temperatures obtained at both 10 cm and 50 cm depths into the clamp face using R (version

4.3.3, R Core Team (2024), Vienna, Austria). Analysis of variance was used to identify differences between means of parameters for each core site, using the following model:

$$Y_{ij} = \mu + site_i + \varepsilon_{ij}$$

Where, Y_{ij}= dependent variable; μ = overall mean; $site_i$ = fixed effect of the i-th coring site (top left shoulder, top right shoulder, top centre and mid centre) and ε_{ij}= residual error.

The measurements of core wet chemistry, NIRS analysis and mycotoxin analysis for the top and mid sections collected during the survey generated a large dataset of approximately 132 variables. The data were scaled by first centring the mean to zero and then dividing by the standard deviation. Where variables demonstrated heteroscedasticity, they were transformed using log. In order to reduce the dimensionality of the dataset objectively, variables (features) were subject to Random Forest analysis (Genuer et al., 2010) in R using the package `randomForest` (Liaw and Wiener, 2002), where features were ranked based on their importance with regards to explaining the variation in the chosen response variable. In most cases this was the concentration of mycotoxins in the silage samples, which included the risk equivalent quantity (REQ ppb). Random Forests were subject to 50,000 randomisations (decision trees), and the results of three Random Forest models were used to provide a mean value for the total explained variance for the particular variable.

Once features had been selected, a series of linear or polynomial models were developed where the Akaike Information Criterion was used to identify the model that explained the largest variance in the response variable with the least terms.

Categorical data from the questionnaire answers were subjected to Kruskal-Wallis rank sum test (Kruskal and Wallis, 1952) in order to assess relationships between silage production and management and the resulting nutritional quality and mycotoxin content. The H test statistic was calculated in R using the function `kruskal.test` in the `stats` package as:

$$H = \frac{12}{N(N+1)} \sum_{i=1}^{g} \frac{R_i^2}{n_i} - 3(N=1)$$

Where, g was the number of groups (e.g. clamps with side sheets and without side sheets = 2 groups), n_i was the number of observations in group i, N was the total number of observations across all groups, R_i was the sum of ranks for the i-th group, and $-3(N=1)$ was an adjustment to allow the H statistic to be compared to the chi-square distribution.

The output provided the X^2 test statistic, degrees of freedom and the test p-value, where $p < 0.05$ was considered significant. Where necessary, *post-hoc* analyses was carried out with a paired samples Wilcoxon test.

3.5. Results

3.5.1. Summary statistics for clamp measurements

3.5.1.1. Clamp measurements: Dimensions

Clamp dimensions varied widely farm to farm, with a minimum height of 1.50 m to a maximum of 6.00 m, with a median of 3.90 m (Table 3-1). Clamp widths ranged from 5.90 to 29.92 m with a median value of 12.5 m. Clamp lengths ranged from 21.5 to 85.5 m with a median of 35.0 m. The area of the clamp faces ranged from 11.6 to 119 m^2, with a median value of 50.8 m^2.

3.5.1.2. Clamp measurements: Compaction density

Compaction density of the silages varied greatly from 157 kg fresh weight (FW)/m^3 to 935 kg FW/m^3 across all four of the core sites (Table 3-1).

The median density ranged from 499 to 662 kg FW/m^3 across all four of the core sites. Mean compaction density of the mid centre cores (666 kg FW/m^3) were approximately 100-150 kg FW/m^3 higher than the density at the top left (496 kg FW/m^3), top centre (561 kg FW/m^3) and top right cores (491 kg FW/m^3; ANOVA: $P < 0.001$).

3.5.1.3. Clamp measurements: Temperature at the core sites

The difference in temperature between the 50 cm and 10 cm depth at each core site ranged from no difference in temperature (0.00 °C) to a maximum difference of 17.0 °C (Table 3-1). At the top left shoulder and top right shoulder the temperature at a 10 cm depth was hotter than at a 50 cm depth. At the top centre and mid centre core sites, temperature at a 10 cm depth was colder than at the 50 cm depth. The greatest difference in temperatures between the 10 cm and 50 cm depth were observed in cores of the top left shoulder, with a maximum difference of 17.0 °C but with a median difference of -1.55 °C. Again, the greatest differences in temperature at the two depths were observed in the shoulder cores in comparison with the top mid and mid centre cores at both 10 cm and 50 cm depths, with a median difference in temperature of no more than -3 °C. There were however no differences (ANOVA: $P > 0.05$) between the mean differences in temperature between 10 cm and 50 cm over the various coring sites.

3.5.2. Summary statistics for silage chemistry

3.5.2.1. Silage chemistry: Fermentation analysis

Near infrared spectroscopy (NIRS) prediction of the DM content of the silage samples (corrected for loss of volatiles) ranged from 219 g/kg to 489 g/kg with a median of 317 g/kg (Table 3-2). Crude protein content predicted by NIRS ranged from 94.0 to 215 g/kg DM with a median of 150 g/kg DM. Mean neutral detergent fibre was 444 g/kg DM and mean acid detergent fibre was 287 g/kg DM. Silage pH ranged from 3.5 to 4.9 with a median of 4.2.

Wet chemical analysis of DM ranged from 219 to 489 g/kg DM, and crude protein from 60.7 to 235 g/kg DM with a median of 136 g/kg DM (Table 3-2). Lactic acid concentration determined by wet chemistry ranged from 24.5 to 188 g/kg DM with a median of 80.5 g/kg DM. Acetic acid concentration ranged from 5.40 to 73.3 g/kg DM with a median of 26.8 g/kg DM. Ethanol concentration ranged from 0.230 to 42.9 g/kg DM with a median of 5.41 g/kg DM and ammonia N ranged from 2.88 to 20.1 % of total N, with a median concentration of 7.66 % of total N.

The ratio of lactic acid to acetic acid, determined by wet chemistry, ranged from 0.334 to 15.0 with a median value of 3.76 (Table 3-2). Lactic acid as a ratio to total VFAs ranged from 0.229 to 13.5 with a median value of 2.93. The ratio of lactic acid to total VFAs and total alcohols determined by wet chemistry, ranged from 0.196 to 8.00 with a median value of 2.02.

3.5.2.2. Silage chemistry: Mycotoxin analysis

A total of 22 out of a possible 46 mycotoxins were detected across the grass silage sampled from across 37 clamps in Great Britain (Table 3-3). The mycotoxin profile of grass silages included DON (present in 14.9 % of total clamps surveyed), *Penicillium* associated mycotoxins such as citrinin (12.2 %), cyclopiazonic acid (4.05 %), PEN (71.6 %), MPA (2.70 %) and roquefortine C (2.70 %), as well as FUS (75.7 %), FUM (6.76 to 9.46 %), ergot alkaloids (2.70 to 8.11 %) and enniatins (present in 20.3 to 27.0 % of total clamps surveyed).

Concentration of total *Penicillium* associated mycotoxins ranged from 8.75 to 10,541 µg/kg with a median of 746 µg/kg, and total *Fusarium* associated mycotoxins ranged from 7.88 to 1,624 µg/kg with a median of 87.5 µg/kg (Table 3-3). Concentration of total *Aspergillus* associated mycotoxins ranged from 7.88 to 10,768 µg/kg with a median of 1,369 µg/kg and total *Claviceps* associated mycotoxins ranged from 4.71 to 2,187 µg/kg with a median of 175 µg/kg. The risk equivalent quantity (REQ) of mycotoxin concentration for all of the seventy-

four silage samples ranged from a minimum of 0.400 to 8,022 µg/kg, with a median value of 295 µg/kg.

Table 3-1. Summary statistics for clamp dimensions, silage compaction density and difference in temperature at a 10 and 50 cm depth at four core sites of the thirty-seven silage clamps across Great Britain.

	N	Min	Max	Median	Mean	SD[1]	SE[2]
Clamp dimensions (m)							
Clamp height	37	1.50	6.00	3.90	3.86	0.874	0.144
Clamp width	37	5.90	29.2	12.5	13.7	5.05	0.830
Clamp length	37	21.5	85.5	35.0	37.6	12.5	2.05
Clamp face area (m^2)	37	11.6	119	50.8	54.1	25.1	4.13
Clamp compaction density (kg fresh weight/m^3)							
Top left shoulder	37	177	833	507	496	168	28.0
Top right shoulder	37	157	787	499	491	164	27.4
Top centre	37	200	909	569	561	183	30.5
Mid centre	37	322	935	662	666	145	23.8
Difference in temperature between the 50 cm and 10 cm depth at each core site[3]							
Top left shoulder	37	0.000	+17.0	-1.55	-1.37	4.62	0.770
Top right shoulder	37	0.000	+13.7	-1.40	-1.88	4.89	0.814
Top centre	37	-0.100	-10.3	-1.90	-2.03	2.74	0.450
Mid centre	37	-0.100	-10.2	-2.90	-2.66	3.82	0.628

[1]SD: standard deviation, [2]SE: standard error, [3]A positive value (+*x)* indicates that the 10 cm depth was +*x* °C warmer than the temperature at 50 cm depth

Table 3-2. Summary statistics for silage fermentation parameters of top and mid samples, obtained by penetrating horizontally into the clamp face at each core site of the thirty-seven silage clamps sampled from across Great Britain.

Silage fermentation profile	N	Min	Max	Median	Mean	SD[2]	SE[3]
Predicted by NIRS (g/kg DM)							
Corrected[1] dry matter (g/kg)	74	238	351	349	351	62.7	7.29
Crude protein	74	94.0	215	150	152	26.4	3.06
Neutral detergent fibre	74	390	500	441	444	25.9	3.01
Acid detergent fibre	74	223	355	289	287	27.4	3.19
Ash	74	62.0	124	85.0	85.3	12.1	1.40
Sugars	74	2.00	80.0	6.00	17.4	20.1	2.34
Lactic acid	74	13.3	173	70.1	78.1	34.2	3.97
Total VFAs	74	2.60	88.7	32.1	33.6	16.2	1.89
pH	74	3.50	4.90	4.20	4.23	0.386	0.045
Determined by wet chemistry (g/kg DM)							
Dry matter (g/kg)	74	219	489	317	325	64.9	7.54
Crude protein	73	60.7	235	136	139	36.0	4.22
Lactic acid	74	24.5	188	80.5	86.6	38.0	4.41
Acetic acid	74	5.40	73.3	26.8	28.8	14.7	1.71
Propionic acid	74	0.064	12.0	0.229	0.955	1.20	0.232
Butyric acid	74	0.051	24.5	0.103	1.11	3.35	0.389
Isobutyric acid	74	0.052	1.09	0.085	0.130	0.184	0.021
Valeric acid	74	0.051	2.99	0.082	0.190	0.500	0.058
Isovaleric acid	74	0.083	2.90	0.190	0.270	0.383	0.044
Hexanoic acid	74	0.051	2.56	0.079	0.188	0.439	0.051
Heptanoic acid	74	0.051	0.173	0.080	0.083	0.020	0.002
Propan-1-ol	74	0.051	13.0	0.150	1.56	2.95	0.343
Propane-1,2-diol	74	0.060	49.5	3.45	6.92	9.19	1.07
Ethanol	74	0.230	42.9	5.41	8.37	8.50	0.989
Ammonia N (% Total N)	73	2.88	20.1	7.66	8.14	2.89	0.339
Lactic[5] : Acetic[6]	74	0.334	15.0	3.09	3.76	2.30	0.267
Lactic : Total VFA's[7]	74	0.229	13.5	2.93	3.52	2.16	0.252
Lactic : Total VFA's + Alcohols[8]	74	0.196	8.00	2.02	2.27	1.33	0.155

[1]Corrected for loss of volatiles by Trouw nutrition, Derbyshire, UK, [2]SD: standard deviation, [3]SE: standard error, [4]CI: 95% confidence intervals,[5]Lactic acid, [6]Acetic acid, [7]Total VFA's include: acetic, propionic, butyric, isobutyric, valeric, isovaleric, hexanoic and heptanoic acids, [8]Alcohols include: propan-1-ol, propane-1,2-diol and ethanol.

Table 3-3: Summary statistics for silage mycotoxin profiles of top and mid samples, obtained by penetrating horizontally into the clamp face at each core site of the thirty-seven silage clamps sampled across Great Britain.

Mycotoxin *(µg/kg)*	Type[1]	N	%[2]	Min	Max	Median	Mean	SD[3]	SE[4]
3-acetyldeoxynivalenol	F	1	1.35	-	-	-	23.3	-	-
Citrinin	P/A	9	12.2	135	1291	334	464	379	126
Cyclopiazonic acid	P/A	3	4.05	5.09	16.5	8.75	10.1	5.81	3.35
Deoxynivalenol	F	11	14.9	37.5	112	44.2	53.7	22.1	6.66
Enniatin A/A_1	F	20	27.0	7.26	85.5	34.3	34.5	20.8	4.66
Enniatin B/B_1	F	15	20.3	2.04	61.6	4.77	11.5	15.6	4.02
Ergocornin(in)e	C	2	2.70	3.84	23.5	13.7	13.7	13.9	9.84
Ergocristin(in)e	C	2	2.70	19.9	151	85.4	85.4	92.6	65.5
Ergocryptin(in)e	C	3	4.05	4.71	779	119	301	418	241
Ergometrin(in)e	C	2	2.70	13.0	32.4	22.7	22.7	13.7	9.67
Ergosin(in)e	C	3	4.05	37.4	700	112	283	363	210
Ergotamin(in)e	C	6	8.11	14.9	974	338	392	378	154
Fumonisin B_1	F	7	9.46	78.6	357	151	207	104	39.4
Fumonisin B_2	F	6	8.11	19.8	86.0	52.1	50.1	26.4	10.8
Fumonisin B_3	F	5	6.76	5.67	34.3	12.9	17.7	11.1	4.97
Fusarenon X	F	1	1.35	-	-	-	1398	-	-
Fusaric acid	F	56	75.7	7.88	306	25.6	75.1	86.4	11.6
Moniliformin	F	3	4.05	5.25	5.74	5.33	5.44	0.263	0.152
Mycophenolic acid	P	2	2.70	36.9	407	222	222	262	185
Penicillic acid	P	53	71.6	33.0	10541	746	1538	2112	290
Roquefortine C	P	2	2.70	17.9	88.3	53.1	53.1	49.8	35.2
Zearalenone	F	9	12.2	9.05	157	91.8	75.6	51.5	17.2
Total *Penicillium* mycotoxins		59	79.7	8.75	10541	746	1462	2028	264
Total *Fusarium* mycotoxins		72	97.2	7.88	1624	87.5	133	207	24.4
Total *Aspergillus* mycotoxins		67	90.5	7.88	10768	746	1369	2006	245
Total *Claviceps* mycotoxins		7	9.46	4.71	2187	175	621	823	311
Risk equivalent quantity		74	100	0.400	8022	295	854	1440	167

[1] Common synthesisers of the specific mycotoxin (*not exclusive*). P = *Penicillium*, A = *Aspergillus*, F = *Fusarium*, C = *Claviceps,* [2]Presence of mycotoxin in grass silage clamp as a percentage of all clamps surveyed, [3]SD: standard deviation, [4]SE: standard error. Only mycotoxins that were detected in the grass silages are shown in this table.

3.5.3. Summary statistics for the silage production questionnaire

3.5.3.1. Questionnaire: Silage clamp structure

Clamp structure across the thirty-seven farms visited, ranged from concrete or sleeper-walled clamps (29) to earth banked clamps (4) and included one pile clamp (Table 3-4). Nearly all the clamps sampled from had a structured floor, where 34 were concrete floored and 2 were

asphalt. Only one silage clamp visited was set on bare ground. The most common top weight used to secure the covering sheets were tyres placed intermittently (16), with only 10 clamps using tyres that were touching all the way around the top of the clamp. Two farms out of the thirty-seven visited had no top weight at all. Thirty-one clamps visited out of the total thirty-seven used sheets to both sides of the clamp where two clamps only used one, and four clamps had no side sheets, at time of visit.

3.5.3.2. Questionnaire: Ensiling methods and clamp management

Out of thirty-three answers obtained for additive application across the thirty-seven clamps, 24 clamps were treated with an additive in comparison to 9 that were untreated (Table 3-5). Out of thirty-four answers obtained for filling the clamp, 19 clamps were filled within a day whereas it took longer than one day to fill 15 clamps. Ninety-one percent of these clamps were rolled again after filling had completed, where 8.8 % were not rolled again. Of those that were rolled again, only four clamps were rolled for a duration greater than an hour after filling. The majority of clamps were compacted without use of a specific compactor machine.

In terms of work delegation, out of thirty-four farmers who answered, 47.1 % carried out all of the harvesting and ensiling work on farm themselves, whereas 29.4 % explained that all the harvest and ensiling work was carried out by a contractor only (Table 3-5). Similarly, 23.5 % of these farms shared the work of harvesting and ensiling between farmer and contractor. Thirty-three answers to the composition of the silage clamps were obtained, revealing that 48.5 % of those contained only a single cut of grass silage, whereas 39.4 % were comprised of two cuts. Only 12.1 % of the thirty-three clamps were comprised of three or more cuts.

Of the thirty-seven clamps sampled from, top weights were used to keep the top sheet tight to the surface of the clamp when open, in 70.3 % of clamps, with 59.5 % of clamps with very little or no effluent present at the base of the clamp (Table 3-5). In contrast 29.7 % of clamps had no top weights present when the clamp had been opened and 40.5 % of clamps had an average to excessive volume of effluent present at the base of the clamp.

3.5.3.3. Questionnaire: Field and harvest management

For the thirty-four answers received regarding field management for the resultant silage produced, 61.8 % was comprised of grass grown in a grass only pasture, in contrast to a mixed pasture, for example including clover or a general multispecies sward (Table 3-6). Seventy-six percent of these pastures received both an artificial fertiliser and slurry application, in comparison to 14.7 % that received only slurry application. Only one farm from the thirty-four responses did not apply any fertiliser (organic or inorganic) to the grass.

Only twenty-five out of the possible thirty-seven responses with regards to environmental stresses that the pasture experienced, were received (Table 3-6). Of these 25 answers, 13 stated that their pasture experienced a period of drought during growth. Twelve responses stated no environmental stress was experienced and no farms reported an impact of flood or pest damage on their pasture used to produce the silage sampled from.

For thirty-four answers received regarding harvesting process, 88.2 % of answers received stated that the grass was left to wilt for either equal to or greater than a 24 hour duration, and the majority of responses (65.7 %) stated that the crop was tedded after mowing (Table 3-6).

Table 3-4. Clamp structure, weight and sheet usage of the thirty-seven silage clamps sampled from across Great Britain.

	Total	N[1]	%N[2]
Clamp structure:			
Domed	17	37	45.9
Flat	20	37	54.1
Roofed	11	37	29.7
Unroofed	26	37	70.3
Concrete/sleeper walled	29	37	78.4
Earth-banked	4	37	10.8
Mixed wall structures	3	37	8.11
Pile (no wall structure)	1	37	2.70
Concrete floored	34	37	91.9
Asphalt floored	2	37	5.41
Field/bare ground	1	37	2.70
Clamp top weight:			
Intermittent tyres	16	37	43.2
Intermittent gravel bags	3	37	8.11
Intermittent mixture (tyres/gravel bags/bales)	4	37	10.8
Tyres touching all the way around	10	37	27.0
Bales	2	37	5.41
No top weight	2	37	5.41
Clamp wall fixing:			
Intermittent tyres	15	37	40.5
Intermittent gravel bags	4	37	10.8
Tyres touching all the way around	9	37	24.3
Gravel bags touching all the way around	6	37	16.2
No wall fixing	3	37	8.11
Clamp sheeting:			
Use of both side sheets	31	37	83.8
Use of sheet on one side	2	37	5.41
No side sheets	4	37	10.8

Table 3-5. Ensiling methods and clamp management of the thirty-seven clamps sampled from across Great Britain.

	Total	N[1]	%N[2]
Ensiling process:			
Additive was applied	24	33	72.7
No additive applied	9	33	27.3
Duration of filling the clamp ≤ 1 d	19	34	55.9
Duration of filling the clamp > 1 d	15	34	44.1
Clamp rolled again after filling	31	34	91.2
Clamp not rolled again after filling	3	34	8.82
Rolling duration ≤ 30 m	19	32	59.4
Rolling duration > 30 m ≤ 1 h	9	32	28.1
Rolling duration > 1 h	4	32	12.5
Compactor used	9	33	27.3
No compactor used	24	33	72.7
All work carried out by farmer only	16	34	47.1
All work carried out by contractor only	10	34	29.4
Work carried out by both farmer and contractor	8	34	23.5
Clamp management:			
One cut in the clamp	16	33	48.5
Two cuts in the clamp	13	33	39.4
Three or more cuts in the clamp	4	33	12.1
≤ 5 days feed out required to remove clamp face	22	31	71.0
> 5 days feed out required to remove clamp face	9	31	29.0
Weights used behind clamp top sheet when open	26	37	70.3
Weights not used behind clamp top sheet when open	11	37	29.7
Little to no effluent present	22	37	59.5
Average effluent present	12	37	32.4
Excessive effluent present	3	37	8.11

Table 3-6. Field and harvest management of the thirty-seven clamps sampled from across Great Britain.

	Total	N[1]	%N[2]
Field management:			
Grass only pasture	21	34	61.8
Grass mixture pasture (clover, multispecies etc.)	13	34	38.2
Artificial fertiliser only applied	2	34	5.88
Artificial fertiliser and slurry/manure	26	34	76.5
Slurry only applied	5	34	14.7
None applied	1	34	2.94
Stress experienced whilst growing:			
Drought	13	25	52.0
Flood	0	25	0.00
Pests	0	25	0.00
None	12	25	48.0
Mowing process:			
Mowing at/before 10am	22	30	73.3
Mowing after 10am	8	30	26.7
Mowing width:			
Full width of the mower	13	28	46.4
Almost full width	14	28	50.0
Narrow width	1	28	3.57
Harvesting process:			
Crop left to wilt for ≤ 24 h	30	34	88.2
Crop left to wilt for > 24 h	4	34	11.8
Crop tedded within 6 hours	14	32	43.8
Crop tedded after 6 hours	7	32	21.9
No tedding	11	32	34.4
Crop rowed up 1-2 h before harvesting	24	32	75.0
Crop rowed up > 2 h before harvesting	8	32	25.0

3.5.4. Analysis of the relationships between silage clamp characteristics, silage chemistry and mycotoxin concentration of the grass silage sampled

3.5.4.1. Identification and selection of silage fermentation parameters associated with mycotoxin contamination

Random forests analysis demonstrated that the variables DM (g/kg), percentage of sugars (predicted by NIRS), acetic acid g/kg DM, lactic acid g/kg DM and ethanol g/kg DM of the silages were the most important ranked features in explaining the variance for the REQ of mycotoxins (logged values; µg/kg) as shown in Figure 3-4.

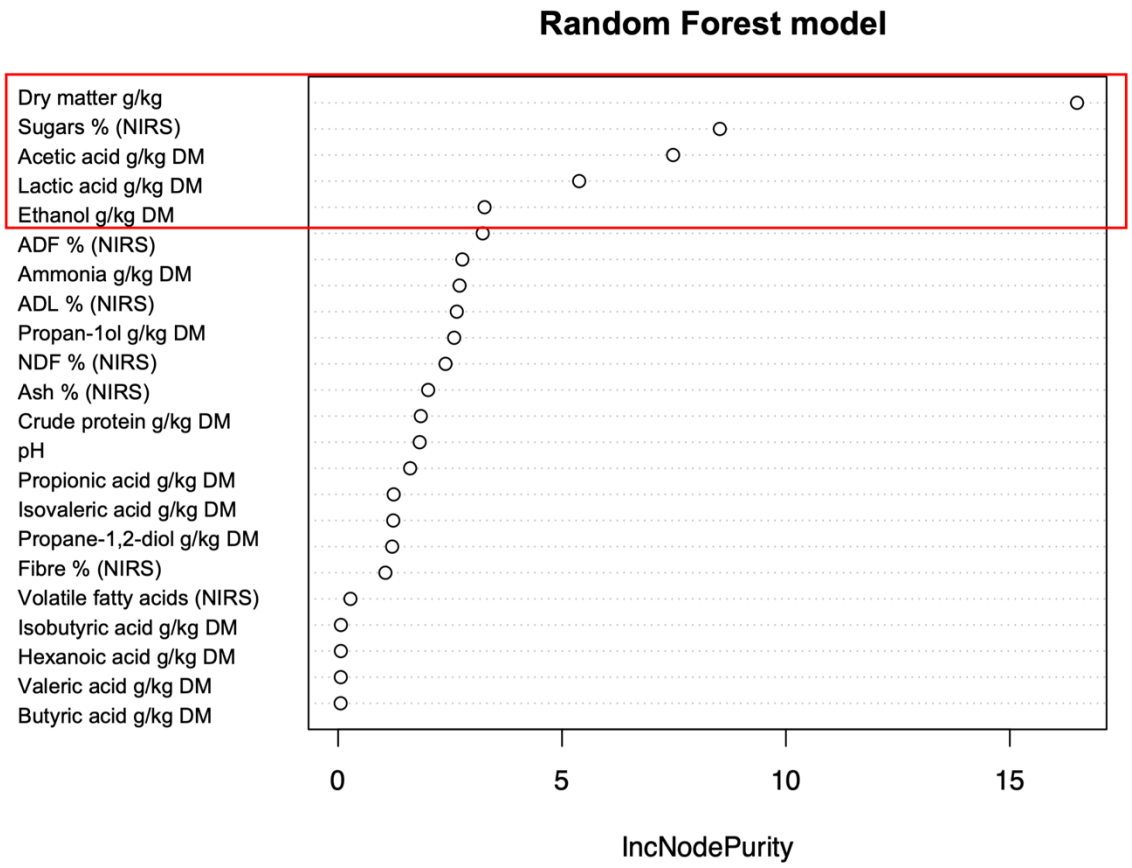


Figure 3-4. Variable importance plot for the silage fermentation parameters (features) with regards to explaining the variance for the Risk equivalent quantity (logged values, µg/kg) of grass silages of thirty-seven silage clamps sampled across Great Britain. The first five features (outlined in red) were selected for further investigation into the relationship.

Linear models of individual features explained between 0.156 (ethanol g/kg DM) and 0.504 (DM g/kg) of the variation ($P < 0.001$) in the REQ of mycotoxins in the seventy-four samples of grass silages (Table 3-7). A linear model incorporating all five of the selected features, was able to explain 0.572 ($P < 0.001$) of the variance in the REQ, however the Akaike Information Criterion value was lower for the polynomial models where feature 4 ([F4] Lactic acid g/kg DM) was squared, demonstrating these models were able to explain the most variance (between 0.572 and 0.582) with the least number of terms involved.

Table 3-7. The amount of variance explained (adjusted R-squared) by a range of models incorporating the five selected features informed by the Random forests analysis, on the resultant risk equivalent quantity of mycotoxin contamination for the thirty-seven clamps sampled from across Great Britain.

Feature [F*x*]	Variance explained[1]	Significance	AIC[2]
Linear models			
[F1] Dry matter g/kg	0.504	< 0.001	301.24
[F2] Sugars % (NIRS)	0.351	< 0.001	321.18
[F3] Acetic acid g/kg DM	0.233	< 0.001	333.51
[F4] Lactic acid g/kg DM	0.273	< 0.001	329.53
[F5] Ethanol g/kg DM	0.156	< 0.001	340.56
[F1] + [F2] + [F3] + [F4] + [F5]	0.572	< 0.001	294.20
Polynomial models			
[F1] + [F2] + [F3] + $[F4]^2$ + [F5]	0.574	< 0.001	293.84
[F1] + [F3] + $[F4]^2$	0.582	< 0.001	290.53

[1]The variance in the risk equivalent quantity explained by the particular model.

[2]AIC = The Akaike Information Criterion, used to identify models that explain the greatest amount of variance with the least terms involved (a lower AIC value indicates a more streamlined model).

Plotting the features demonstrated a negative correlation between DM content and the REQ (Figure 3-5), and a positive correlation between the concentrations of acetic (Figure 3-6) and lactic acids (Figure 3-7) in the silage samples across the thirty seven clamps. There was a negative correlation between sugars (%, predicted by NIRS), and REQ, and a positive correlation between ethanol g/kg DM and REQ.

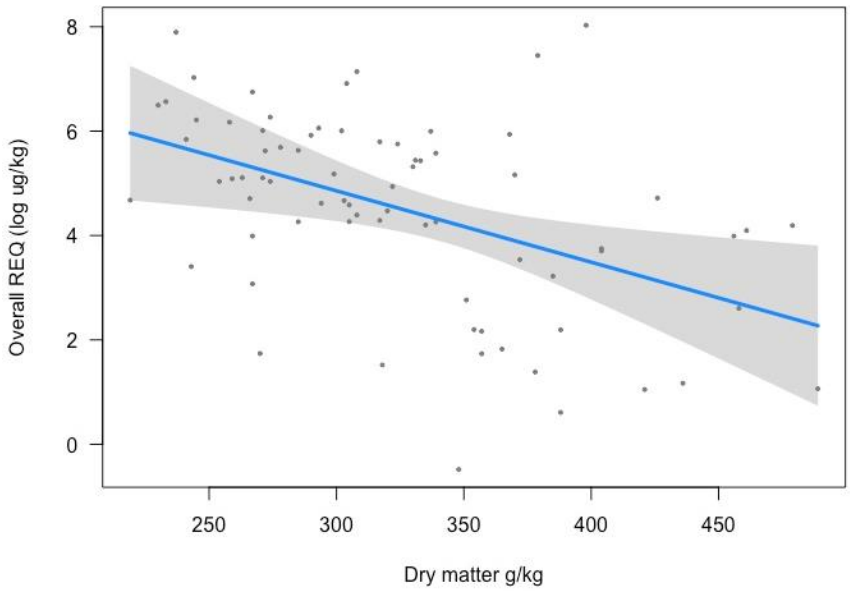


Figure 3-5. Linear regression for dry matter content (g/kg) of the grass silage samples against the risk equivalent quantity (REQ; log µg/kg) of mycotoxins in the seventy-four grass silage samples across thirty-seven clamps in Great Britain.

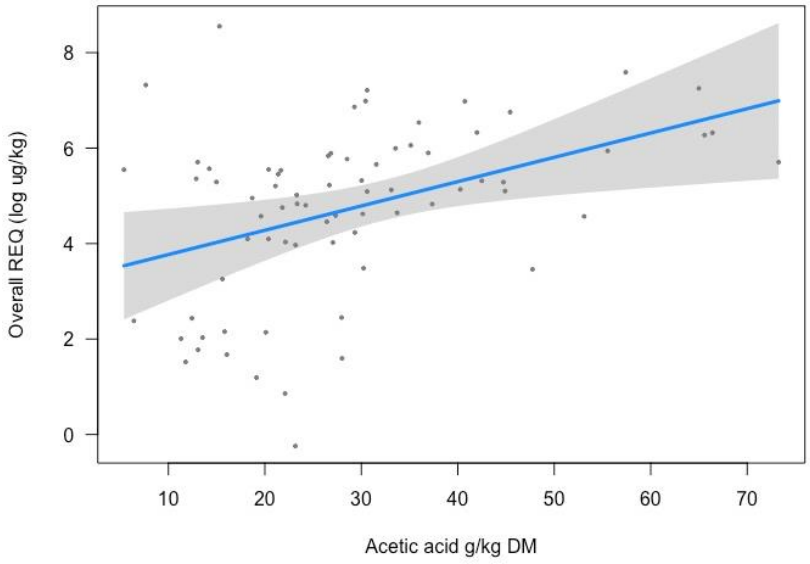


Figure 3-6. Linear regression for acetic acid concentration (g/kg DM) of the grass silage samples against the risk equivalent quantity (REQ; log µg/kg) of mycotoxins in the seventy-four grass silage samples across thirty-seven clamps in Great Britain

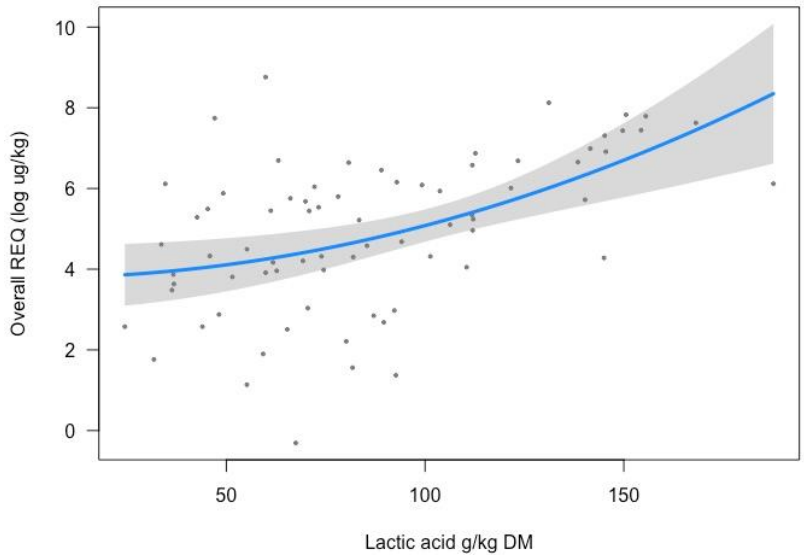


Figure 3-7. Polynomial regression for lactic acid concentration (g/kg DM) of the grass silage samples against the risk equivalent quantity (REQ; log µg/kg) of mycotoxins in the seventy-four grass silage samples across thirty-seven clamps in Great Britain

3.5.4.2. Relationship of silage chemistry with silage clamp structure

Aspects of silage clamp structure, such as a domed or flat clamp structure, roofed or unroofed, did not demonstrate any relationship with DM content, acetic acid concentration or lactic acid concentration of the silage (Table 3-8). There were also no relationships found with concentrations of fungal genera associated mycotoxins. Use of side sheets was associated with a lower concentration of acetic acid concentration of the silage (*Kruskal- Wallis* $\chi 2$ = 6.49, *d.f.* = 2, P = 0.039), where mean acetic acid concentration for use of side sheets was 26.7 g/kg DM, compared with 41.2 g/kg DM in silage from clamps without side sheets. No other associations for use of side sheets were found.

Table 3-8. Significance of associations between silage clamp structure parameters, fermentation parameters identified as important in the determination of mycotoxin content and mycotoxin concentrations associated with particular fungal genera.

	Significance of silage clamp structure parameters[1]		
	Domed or flat[2]	Roofed or unroofed[3]	Side sheets[4]
	n = 74	*n = 74*	*n = 74*
Dry matter g/kg	0.357	0.249	0.292
Lactic acid g/kg DM	0.854	0.723	0.087
Acetic acid g/kg DM	0.536	0.261	0.039
Lactic acid : acetic acid	0.448	0.219	0.433
Total *Penicillium* mycotoxins µg/kg	0.777	0.462	0.319
Total *Fusarium* mycotoxins µg/kg	0.522	0.772	0.085
Total *Aspergillus* mycotoxins µg/kg	0.983	0.428	0.304
Alltech© REQ log_{10}(µg/kg)	0.875	0.401	0.198

[1] Significance (P-value) was calculated using the Kruskal-Wallis rank sum test. All unknown values were removed prior to analysis.

[2] Clamp top shape: 2 groups: Domed shape, or flat topped.

[3] Clamp housing: 2 groups: Covered by a roof or not.

[4] Use of plastic sheets to both sides of the clamp: 2 groups: yes or no.

3.5.4.3. Relationship of silage chemistry, ensiling methods and clamp management

Type of additive used (obligate heterofermentative (ObHe) or obligate homofermentative (ObHo) and/or facultative heterofermentative (FaHe)), demonstrated no association with DM content, or lactic acid content (Table 3-9). There was a difference demonstrated between the additive groups with a higher mean (*Kruskal-Wallis* $\chi 2$ = 5.82*, d.f.* = 1, P = 0.016) for ObHe groups for the concentration of acetic acid (34.2 g/kg DM), and subsequently a lower ratio (3.55) in comparison to ObHo and FaHe groups (22.1 g/kg DM and 4.25, respectively). There were no differences between additive group and mycotoxin concentration, or REQ.

Duration of filling was classified as filled in one day or filled in more than one day (Table 3-9). No differences between groups were found for any of the fermentation parameters or mycotoxin concentration, or REQ.

Delegation of work was split into three groups, either work was completely carried out by the farmer only, contractor only, or a combination of the two (Table 3-9).The DM content, acetic acid concentration, and lactic acid to acetic acid ratio were not different between the three groups, however lactic acid concentration did differ. Lactic acid g/kg DM were highest (*Kruskal-Wallis chi squared* = 6.9139 *d.f.* = 2, P = 0.032) for the group where the work was

carried out exclusively by the farmer (98.1 g/kg DM), and lowest for the exclusively contractor group (75.1 g/kg DM). The mixed group was in between but was not different from the contractor only group or the farmer only group (77.3 g/kg DM).

Silage clamps that had either been composed of one cut (single cut) or more than one (multicut) did not demonstrate any differences between any of the fermentation parameters or mycotoxin concentrations associated with a particular fungal genus (Table 3-9)..

Feedout rate was grouped by either ≤ 5 days to feed out the whole front face of the clamp or > 5 days and demonstrated a difference between groups with a higher DM (*Kruskal-Wallis* $\chi 2$ = 1.5414, *d.f.* = 1, P = 0.214) in clamps where the feedout rate was > 5 days (369 g/kg) in comparison to ≤ 5 days (322 g/kg; Table 3-9). There were no differences found between groups for any of the other parameters or mycotoxin concentrations.

For the duration of the clamp being open, weights were either used to keep the top sheet flat across the clamp top, or no weights were used. There were differences in DM content, *penicillium* associated mycotoxin concentration, *aspergillus* associated mycotoxin concentration and REQ between the two groups. The DM content was higher in clamps with weights (336 g/kg; *Kruskal-Wallis* $\chi 2$ = 4.76, *d.f.* = 1, P = 0.029), and subsequently lower in *penicillium* (966 µg/kg; *Kruskal-Wallis* $\chi 2$ = 5.93, *d.f.* = 1, P = 0.015) and *aspergillus* associated mycotoxins (1055 µg/kg; *Kruskal-Wallis* $\chi 2$ = 4.44, *d.f.* = 1, P = 0.035). Clamps without weights had a mean DM content of 298 g/kg, and *penicillium* and *aspergillus* associated mycotoxin concentrations of 1637 µg/kg and 1675 µg/kg, respectively. The REQ values for the two groups also differed, with a higher REQ (*Kruskal-Wallis* $\chi 2$ = 6.77, *d.f.* = 1, P = 0.009) for the clamps without weights (2.62 $\log_{10}$ µg/kg), in comparison to clamps with weights (1.94 $\log_{10}$ µg/kg).

Table 3-9. Significance of associations between ensiling methods and clamp management, fermentation parameters identified as important in the determination of mycotoxin content and mycotoxin concentrations associated with particular fungal genera.

	Significance of ensiling methods and clamp management[1]					
	Additive type[2] *n = 38*	Duration of filling[3] *n = 68*	Farmer vs contractor[4] *n = 68*	Single vs multicut[5] *n = 66*	Feedout rate[6] *n = 62*	Weights in use[7] *n = 74*
Dry matter g/kg	0.357	0.224	0.231	0.863	0.021	0.029
Lactic acid g/kg DM	0.997	0.648	0.032	0.547	0.476	0.786
Acetic acid g/kg DM	0.014	0.135	0.092	0.681	0.226	0.086
Lactic acid : acetic acid	0.016	0.051	0.797	0.807	0.988	0.554
Total *Penicillium* mycotoxins µg/kg	0.141	0.503	0.225	0.733	0.174	0.015
Total *Fusarium* mycotoxins µg/kg	0.114	0.509	0.057	0.492	0.332	0.732
Total *Aspergillus* mycotoxins µg/kg	0.079	0.489	0.253	0.888	0.214	0.035
Alltech© REQ log_{10}(µg/kg)	0.309	0.338	0.422	0.719	0.185	0.009

[1] Significance (P-value) was calculated using the Kruskal-Wallis rank sum test. All unknown values were removed prior to analysis.

[2] When applied, additive type: 2 groups: Obligate heterofermentative, or obligate homofermentative and/or facultative heterofermentative additive application

[3] Time taken to fill the clamp upon ensiling: 2 groups: ≤ 1 day, or > 1 day.

[4] All work carried out by farmer or contractor: 3 groups: exclusively farmer, exclusively contractor, or mixed.

[5] Composition of the clamp: 2 groups: only 1 cut (single), or > 1 cut (multicut).

[6] Rate to feedout across the whole clamp face: 2 groups: ≤ 5 days, or > 5 days.

[7] Weights still in use on top of the clamp, when open: 2 groups: yes, or no

3.5.4.4. Relationship of silage chemistry with field and harvest management

Drought stress was classified into two groups, where the sward had either experienced periods of drought at any time throughout growth or had not (Table 3-10). There was a difference between the groups with a lower DM content (*Kruskal-Wallis* $\chi 2$ = 4.20, *d.f.* = 1, P = 0.040) for silage that had been formed from a sward that experienced drought (309 g/kg) in comparison to a sward that had not (344 g/kg). Acetic and lactic acid concentrations did not differ between groups but *penicillium* and *aspergillus* associated mycotoxins did (Table 3-10). *Penicillium* (*Kruskal-Wallis* $\chi 2$ = 4.98, *d.f.* = 1, P = 0.026) and *aspergillus* (*Kruskal-Wallis* $\chi 2$ = 9.78, *d.f.* = 1, P = 0.002) associated mycotoxins were both higher in silages formed from swards that had experienced drought (1863 µg/kg, and 1940 µg/kg respectively) in comparison to swards that had not (295 µg/kg and 319 µg/kg, respectively). Similarly the REQ was higher (*Kruskal-Wallis* $\chi 2$ = 8.83, *d.f.* = 1, P = 0.003) in silage formed from swards that had experienced drought stress (2.45 log_{10} µg/kg) compared with that which had not (1.52 log_{10} µg/kg; Table 3-10).

Sward type was formed of two groups, silage produced for a grass only sward or silage produced from a grass mixture (Table 3-10). There were differences in DM content, acetic acid and lactic acid to acetic acid ratio between the two groups with DM higher (*Kruskal-Wallis* $\chi 2$ = 5.84, *d.f.* = 1, P = 0.016) in silage formed from mixed swards (353 g/kg) compared with grass only swards (300 g/kg). Acetic acid concentration was higher (*Kruskal-Wallis* $\chi 2$ = 16.0, *d.f.* = 1, P < 0.001) in silage made from grass only swards (36.9 g/kg DM) compared with mixed swards (20.5 g/kg DM). In consequence, lactic acid to acetic acid ratio was therefore lower (*Kruskal-Wallis* $\chi 2$ = 7.50, *d.f.* = 1, P = 0.006) in silage produced from grass only swards (2.76) in comparison to sward mixtures (4.62).

Silage produced from grass only swards were higher in *Aspergillus* associated mycotoxins (1,555 µg/kg; *Kruskal-Wallis* $\chi 2$ = 4.24, *d.f.* = 1, P = 0.039) compared to those of a grass mixture (736 µg/kg). There was a trend for *Penicillium* associated mycotoxins (*Kruskal-Wallis* $\chi 2$ = 2.77, *d.f.* = 1, P = 0.096), and *Fusarium* associated mycotoxins (*Kruskal-Wallis* $\chi 2$ = 3.77, *d.f.* = 1, P = 0.052) to be higher in grass only silage (1,462 µg/kg and 183 µg/kg, respectively), compared with silage produced from mixed swards (729 µg/kg and 69.2 µg/kg, respectively; Table 3-10). Likewise, there was a trend for the REQ to be higher (Kruskal-Wallis $\chi 2$ = 3.26, df = 1, p-value = 0.071) in silages produced from grass only swards (2.28 $\log_{10}$ µg/kg) in comparison to mixed swards (1.70 $\log_{10}$ µg/kg).

Table 3-10. Significance of associations between field harvest and management parameters, fermentation parameters identified as important in the determination of mycotoxin content and mycotoxin concentrations associated with particular fungal genera.

	Significance of field harvest and management[1]	
	Drought stress[2]	Sward type[3]
	n = 50	*n = 32*
Dry matter g/kg	0.040	0.016
Lactic acid g/kg DM	0.140	0.437
Acetic acid g/kg DM	0.207	< 0.001
Lactic acid : acetic acid	0.861	0.006
Total *Penicillium* mycotoxins µg/kg	0.026	0.096
Total *Fusarium* mycotoxins µg/kg	0.351	0.052
Total *Aspergillus* mycotoxins µg/kg	0.002	0.039
Alltech© REQ log_{10}(µg/kg)	0.003	0.071

[1] Significance (P-value) was calculated using the Kruskal-Wallis rank sum test. All unknown values were removed prior to analysis.

[2] Any periods of drought experienced by the crop when in the field: 2 groups: yes, or no.

[3] The contents of the sward and silage (e.g. multispecies swards): 2 groups: grass only, or grass mixture.

3.6. Discussion

3.6.1. Methodology of data collection

Silage samples were collected from thirty-seven farms, and there was large variation in the structure of the silage clamps, management, ensiling methods and silage chemistry, representing a wide range of silage clamps in Great Britain. Data was originally collected from fifty-six farms, however nineteen were removed due to a large amount of incomplete data. In order to improve the strength of survey additional farms should be included, and the survey carried out over a period of 2 years to collect information across seasons. More samples of silage from Wales and the inclusion of Northern Ireland would also provide a wider geographical range of samples, and environmental conditions as this can impact on the sward and subsequent silage (Skladanka et al., 2013).

Silage cores were taken from the clamp face at the time of visit, and it was not possible to standardise the duration of exposure of the face to air. Some other studies undertaken on baled silage exposed the silage to air just before feeding (O'Brien, 2010). This may have influenced the fermentation parameters associated with aerobic spoilage of the silage, such

as pH, ammonia N concentration, or even the temperature of the core sites and the subsequent mycotoxin concentration (Uriarte et al., 2001). In order to mitigate this, future studies should either sample immediately after removal of a 50 cm depth into the face of the clamp or include a silage core site taken vertically into the clamp from the top of the clamp at specific depths, where oxygen is less likely to have proliferated.

3.6.2. Summary statistics of silage clamp measurements, silage chemistry and silage management practices

Clamp dimensions varied greatly across farms visited, implying that there was also consequent variation in ensiling methods, clamp management and farm herd size; increasing the applicability of conclusions drawn to grass silages produced in Great Britain. Means for clamp dimensions of width, height and length were similar to that reported by (Davies, 2018) during a survey on twenty grass silage clamps across England (Table 3-11).

Table 3-11. Comparison of mean clamp dimensions from a farm survey carried out by (Davies, 2018) and the results for this study.

Mean clamp dimensions	Davies (2018), *n = 20*	This study, *n = 37*	Difference
Width (m)	12.8	13.7	0.90
Height (m)	2.96	3.86	0.90
Length (m)	29.2	37.6	8.40
Clamp face area (m^2)	31.8	54.1	22.3
Silage density (kg FW/m^3)	613	554	59.0

The mycotoxin profile of the grass silage was absent of aflatoxins, T2-toxin, and ochratoxins, with the prominence of FUM, enniatins, ergot alkaloids and PEN, as reported by others (Manni et al., 2022; O'Brien et al., 2008; Skladanka et al., 2013). Beauvericin, MPA and roquefortine C, were most prominent in the grass silage sampled by Manni et al. (2022), however much lower rates of incidence were found in the current study, with no beauvericin present, and both MPA and roquefortine C only present in 2.70 % of the clamps surveyed. These differences may be explained by species composition of the silage, as the Finnish grass silages sampled by Manni et al. (2022), were comprised of mixtures of majority meadow fescue, timothy grass and legumes, in comparison to this study where 61.8 % of samples were reported as grass only. It is known that different species of grasses are more resistant to certain fungal infection in-field (Skladanka et al., 2013) and the inclusion of leguminous crops can alter the fermentation of silage through buffering effects (Bolsen et al., 1996; Playne and McDonald,

1966). Penicillic acid was present in 71.6 % of silage clamps in the current survey, indicating that despite lower roquefortine C and MPA presence, *Penicillium* fungi were still present.

The EU regulated mycotoxins that were present in the study were DON and ZEA, occurring in 14.9 % and 12.2 % of the silage clamps, respectively. Where DON and its derivative metabolite of 3-acetyldeoxynivalenol were present, concentrations were low (maximum of 112 µg/kg) in comparison to the 2,000 µg/kg, (on an 88% DM basis), that is the threshold by the EU for complete feed destined for dairy calves (European Union (EU), 2006). Where present, ZEA was also lower (maximum of 157 µg/kg DM) in comparison to an EU threshold of 500 µg/kg for ZEA contamination in complete feed destined for dairy calves (European Union (EU), 2006), although this difference is not as large as DON and therefore may have potential chronic animal health issues if consistently fed (Rivera-Chacon et al., 2024).

There was considerable variation in the silage mycotoxin concentration in the current study, which may be attributed to the variability in sampling method, as well as the mycotoxin content across a clamp (McElhinney et al., 2016b). Despite bulking cored samples and sampling from four sites to obtain a more representative and homogeneous sample, there is still the possibility that the overall mycotoxin load was under or overestimated due to the reduction in scale from a clamp of approximately 2,000 m^3 of silage to a subsample of approximately 400 g fresh weight during analysis of mycotoxin content in the laboratory. McElhinney et al. (2016b) suggested that the variability in mycotoxin results from sampling pit silage is moderate in comparison to sampling baled silage, where a greater number of sampling replicates are required to reduce the error. McElhinney et al. (2016b) also hypothesised that a greater mixing occurs upon filling a silage clamp or pit as opposed to producing bales, however this would only concern mycotoxins that are already present on the freshly cut grass. The current study demonstrated a large proportion of mycotoxins associated with *Penicillium* and/or *Aspergillus* fungi, which are fungi often associated with aerobic spoilage (O'Brien, 2010) and so it may be possible that these fungi were unevenly distributed throughout the clamp as they may not be present at the ensiling stage. Nevertheless, four silage cores are generally recommended for representative sampling for fermentation analysis (McElhinney et al., 2016b), and cores were taken horizontally into the clamp face, regardless of the presence or absence of visible mould. The overestimation of contamination for the whole clamp was therefore reduced (McElhinney et al., 2016b) in comparison to studies where visibly spoiled areas of silage were targeted for mycotoxin analysis (Manni et al., 2022).

Variability of mycotoxin content within clamps is particularly true for those comprised of more than one cut of grass silage, where silage composition, field conditions, maturity of the sward at harvest and application of fertiliser and additives can all influence the DM and fermentation

profile (Borreani et al., 2018; Ferris et al., 2022; Kim et al., 2021). If mycotoxin risk is associated with lower DM silages, as suggested by the results of this survey, then there could be a great variability in mycotoxin vertically load from one area of a clamp to the next, vertically. In both 2022 and 2023, farms were visited during the months of March and April where farmers were actively feeding out from silage and were either approximately halfway or no more than three quarters of the way through the entire clamp, reducing the likelihood of obtaining samples from the very beginning or very end of the silage clamp, where the risk of air entry may have been greater due to the likelihood of poorer compaction (Borreani et al., 2018).

Aerobic spoilage has been correlated with an increase in fungal proliferation (Le Cocq et al., 2020), and is particularly susceptible at the shoulders of a clamp. A study by Franco et al. (2022) considered a silage density of 424 kg FW/m^3 as loosely compacted, and 583 kg FW/m^3 as tightly compacted silage. Furthermore, a study by Snelling et al. (2023) assigned a density of 500 kg FW/m^3 as high compaction and 333 kg FW/m^3 as low compaction. The median density of the silage in the current study was 507 and 499 kg FW/m^3 for the left and right shoulders, respectively, suggesting that on average the shoulders were well compacted, reducing the likelihood of an aerobic spoilage induced difference in fungal proliferation. This is supported by the lack of heating observed in the median difference of 1.37 °C ± 0.770 and -1.88 °C ± 0.814 at the 10 cm depth compared with the 50 cm depth into the clamp face. Maximum differences of an increase of 17 °C and 13.7 °C in the left and right shoulder at 10 cm compared with 50 cm may be explained by extreme instances of heating on two farms in the study (including one farm where the left shoulder had experienced slippage).

3.6.3. Relationship between silage fermentation features and the REQ

The current study demonstrated that silages with a low DM were associated with a higher REQ. Losses of silage DM can occur during harvest, the fermentation phase and again upon opening upon exposure to air (Borreani et al., 2018). The presence of both high levels of lactic and acetic acid in silage at opening is indicative that the silage has experienced a slower fermentation, allowing for the possibility of DM losses to spoilage organisms such as *Clostridia* (Wróbel et al., 2023). A slower fermentation may also allow the initial growth of fungi in areas where oxygen was still present early-on in the fermentation phase (Grahl et al., 2012). Additionally, DM is lost from silage through the production of acetic acid, ethanol and CO_2 by obligate heterofermentative lactic acid bacteria, of which acetic acid and ethanol were both found to be positively correlated with the REQ (McDonald et al., 1991). Acetic acid is also correlated with a slower acidification rate of silage due to its higher pKa in comparison to lactic acid (McDonald et al., 1991). Silage with a higher sugar content was associated with a lower

REQ, supporting the hypothesis that silage that has undergone a more rapid acidification has a lower mycotoxin load (McDonald et al., 1991).

A reason for this interaction may be that the slower rate of fermentation allows for the growth of bacteria such as *Clostridia* and *Enterobacteriaceae* which may compete with fungi as well as the epiphytic LAB for fermentable carbohydrates present in the forage (McDonald et al., 1991; Wróbel et al., 2023). Mycotoxins can be synthesised to provide fungi with a competitive advantage over other microorganisms (Losada et al., 2009). In particular, PEN was found in almost 72 % of the total clamps sampled and is known to exert antibiotic properties (Geiger and Conn, 1945) and FUS, found in 76 % of total clamps plays a role in disrupting bacterial quorum sensing (Venkatesh and Keller, 2019). Additionally, there are a range of compounds synthesised by LAB present, with antifungal properties that may in turn trigger mycotoxin production in response (Sadiq et al., 2019; Wang et al., 2012). Another suggestion for mycotoxin production during the fermentation phase is that depletion of oxygen and an increase in the acidity of the immediate environment may exert stress on the fungi -mycotoxins may be synthesised to remediate their changing environment, in order for survival (Vylkova, 2017).

Information on the initial DM content of the forage prior to ensiling would have enabled the determination of DM loss and its relationship with the REQ. The longer the duration of the wilt, the longer the period of time that a forage is in contact with field fungi (Hodulíková et al., 2016). Having been mown, the forage will have released plant metabolites and enzymes which could trigger the production of mycotoxins by epiphytic fungi (Pusztahelyi et al., 2015). Moreover, some mycotoxins are also known to be synthesised to protect fungal spores from UV radiation (Keller, 2019), and so could lead to an increase in the original mycotoxin load brought in from the field, before ensiling.

The acetic acid concentration of grass silage is associated with aerobic stability, via a reduced incidence of the growth of filamentous fungi and yeasts (Danner et al., 2003). However, the positive relationship between acetic acid and the REQ supports that mycotoxin production occurs during the fermentation stage, as silage with a higher acetic acid concentration that would be expected to be more aerobically stable, still presented with mycotoxin contamination in the current study. There was also overall no indication of excessive aerobic spoilage in the majority of the clamps sampled from, as indicated by the lack of clamp face heating, and a relatively high compaction density, yet all samples contained at least one mycotoxin. Ethanol, though produced by yeasts, is also a product of obligate heterofermentative LAB fermentation (McDonald et al., 1991). The lack of evidence of yeast growth in the silages, along with the positive association between acetic acid and the REQ, suggests that mycotoxin production is

more heavily influenced by the fermentation of the grass silage than aerobic spoilage at opening.

Future studies should determine the impact of aerobic exposure on the REQ. Additionally, the extent to which the REQ can be reduced through directing the fermentation towards that of obligate homofermentative and facultative heterofermentative LAB as opposed to obligate heterofermentative LAB should be investigated.

3.6.4. Relationship between silage chemistry and clamp structure

The use of side sheets when ensiling, was associated with a lower mean concentration of acetic acid, at 26.7 g/kg DM compared with silage that had not been ensiled with side sheets, with a mean of 41.2 g/kg DM (Table 3-8). A silage clamp without side sheets is more at risk of oxygen ingress into the clamp when open and so yeasts may proliferate, leading to the production of acetic acid (McDonald et al., 1991). However, there was no evidence to suggest that the majority of clamps suffered excessive yeast activity or aerobic spoilage, indicated by the lack of clamp face heating observed. The resulting acetic acid differences are therefore likely to have been driven during the fermentation phase. An establishment of a low pH rapidly during the fermentation process may have been less efficient in circumstances where side sheets were missing, resulting in a higher proportion of acetic acid present at the point of sampling in these cases. In contrast, greater oxygen ingress and a higher acetic acid concentration would have been expected to influence the mean REQ in clamps with side sheets in comparison to those without, but this was not so. However, the number of silage clamps sampled from without adequate side sheets was only 6 in comparison to 31 with side sheets. No relationship was found between the concentration of mycotoxins, grouped by their most common fungal producer, and clamp structure, whether a domed or flat, roofed or unroofed or side sheeted clamp.

3.6.5. Relationship between silage chemistry, ensiling methods and clamp management

Silages that had received obligate heterofermentative additives were 12.1 g/kg DM higher in acetic acid compared to those treated with obligate homofermentative or facultative heterofermentative additives, as was expected (S. J.W.H. Oude Elferink et al., 2001; Ranjit and Kung, 2000). However there was no difference in lactic acid concentration, which may be explained by all LAB groups being able to produce lactic acid, although the lactic acid to acetic acid ratio differed between additive treated groups, a finding in agreement with Wang et al. (2014) Where possible the additives were identified by brand and manufacturer, but this

proved challenging to collect accurately for each farm and so the sample size for the two groups includes only nineteen out of the total of thirty-seven silage clamps. A larger sample size may detect a difference in lactic acid concentration between additive types, although many commercially available additives often contain a mixture of LAB of differing fermentation pathways (Muck et al., 2018).

Whether the silage clamp was filled in one day or two was not associated with a difference in the DM, lactic acid, acetic acid, or mycotoxin content of the silage. There was a trend (P = 0.051) for a higher proportion of lactic to acetic acid in silage from pits that had been filled in more than one day (*n = 15,* mean: 4.56) in comparison those that were filled within one day (*n = 19*, mean: 3.30). A manner of factors could have influenced filling time, including the starting time of mowing the sward, the wilting time required, the volume of forage to be ensiled and the clamp dimensions, the duration of compaction required, and even the distance between the silage clamp and the sward. Future studies should aim to elucidate how long the clamp is left unsheeted upon arrival of the first batch of forage for filling, as a better measure of prolonged exposure to air pre-ensiling.

Interestingly, a lower mean lactic acid concentration of 23 g/kg DM, was associated with silages where the harvest and ensiling had been carried out by a contractor. There was also a trend (P = 0.057) for a higher mean concentration of *Fusarium* associated mycotoxins where a contractor was reported to have carried out all the work (*n = 10,* mean: 160 µg/kg) compared to where the farmer had carried out all the work (*n = 16,* mean: 118 µg/kg) or a mixed workload between both the contractor and the farmer (*n = 8,* mean: 80 µg/kg), but these differences are biologically minimal and could be down to sampling error, particularly with regard to the small *n* value for each group. Furthermore, there was no effect on the concentration of the REQ or other mycotoxins associated with *Aspergillus* or *Penicillium*, and there is little information from the literature comparing the effect of contractors vs farmers on silage quality and mycotoxin load.

No differences were found between lactic and acetic acid concentrations or DM content of clamps either composed of one cut or multiple cuts, nor were there any effects on the REQ, or mycotoxins associated with a particular fungal genus. This was not surprising as there was no specification as to what cut had been included in the multicut silage group and so single cut silages ranged from 1st to 4th cut, and multicut silages could have been a combination of any of the four cuts. A multicut clamp will experience periods of opening post ensiling to facilitate the second round of filling, which could lead to DM losses (Borreani et al., 2018) however, a difference was not found in the current study.

A reported feed out rate of five days or less was associated with a lower DM (322 g/kg) than farms with a feedout rate greater than five days (368 g/kg), despite these clamp faces likely being exposed to oxygen for longer periods of time, which could initiate a loss of DM (Borreani et al., 2018). The range of reported days was between 2 and 8 days, with grouping set at 2 – 5 or 6 - 8. As the silages were well compacted, as demonstrated by the mean compaction, then overall oxygen ingress over a maximum of 8 days may not have been sufficient to observe any biologically relevant impact on the DM due to aerobic spoilage (Borreani et al., 2018).

Farms where top weights were used to keep the top sheet tight to the silage had a higher mean DM content of around 336 g/kg, 38 g/kg higher than farms without. Moreover, the presence of a top weight was also associated with a 671 µg/kg lower concentration of *Penicillium* associated mycotoxins, and 620 µg/kg lower *Aspergillus* associated mycotoxins. The mean REQ was also higher in silage from clamps without a top weight present (mean: 416 µg/kg) in comparison to clamps with a top weight present (mean: 87.0 µg/kg). The mycotoxins synthesised by these two fungal genera are usually classified as predominantly storage-formed mycotoxins (Storm et al., 2014) supporting that aspects of clamp management influence the production of mycotoxins in grass silages (Dell'Orto et al., 2015; Wambacq et al., 2016). Whether these mycotoxins form during the fermentation phase or during periods of aerobic exposure upon opening the clamp, remains to be clarified.

3.6.6. Relationship between silage chemistry and field and harvest management

A lower mean DM content was recorded in silage (309 g/kg) that had been reported to have experienced drought stress in the field compared to those that experienced no stress (344 g/kg), indicating drought stress may have led to a greater loss of DM during ensiling, although the DM of the grass at ensiling is not known. Stress from drought can render a plant susceptible to pathogenic organisms (Szczepaniec and Finke, 2019), which may increase the load of fungi and unfavourable bacteria that are brought in with the silage. An increase in competition between spoilage organisms and epiphytic LAB during the ensiling may lead to a slower rate of pH decline and a greater loss of DM as a result (Zi et al., 2022).

If drought and a subsequently higher ambient temperature was experienced at the time of cutting, the initial mycotoxin load of the sward may have been increased. Skladanka et al. (2013) demonstrated that cutting time of the sward influenced the *Fusarium* mycotoxins of DON and zearalenone concentrations in grass silages, with cooler temperatures associated with lower incidences. In the current study the total *Fusarium* associated mycotoxin concentrations were not different between the swards that had (168 µg/kg) or had not experienced drought (85.3 µg/kg). However, it is of interest to note that there was a mean

concentration of 1,940 µg/kg total *Aspergillus* associated mycotoxins demonstrated in silage made from swards that had experienced drought stress (*n = 13*), which was 1,621 µg/kg higher than that made from silage that had experienced no stress (*n = 12)* although there are no reports in the literature of an effect of heat stress on the subsequent concentration of these mycotoxins in grass silage.

Similarly, a mean concentration of 1,863 µg/kg total *Penicillium* associated mycotoxins was observed in silage that was reported to have experienced drought stress, 1,568 µg/kg higher than those that had no stress. This suggests possibly two different mechanisms that the drought experienced by the sward may impact on the mycotoxin concentration of a silage. Firstly, that the droughted sward leads to a compromised fermentation due to a larger fungal load at harvest (Zi et al., 2022), or a period of drought leads to the stress of fungi in the field, leading to the formation of mycotoxins in the field, which are then brought into the silage clamp (Hodulíková et al., 2016; Skladanka et al., 2013). Both *Penicillium* and *Aspergillus* are generally classified as fungal genera that produce mycotoxins during the post-harvest or storage phase (Storm et al., 2014). However, both are prominent in the soil (Hill, 1972; Nji et al., 2023) and it may be possible that these mycotoxins are produced in-field under periods of environmental stress, although there is a lack of information on this in the literature, and further studies are warranted.

Swards that were reported to be composed of grass only had a lower lactic to acetic acid ratio which was associated with a lower DM content. This was correlated with an increase in the concentration of *Aspergillus* associated mycotoxins, and a trend for higher total *Fusarium* and *Penicillium* associated mycotoxins, as well as a trend for a higher REQ. This supports the previous findings of this study that a decrease in DM content and increase in acetic acid is correlated with an increase in silage mycotoxin concentration. The composition of the sward will impact heavily on the interactions between plant and fungi in the field (Mhuireach et al., 2022), as well as the direction of fermentation of the silage. This can be affected by both the resistance of the particular sward variety to certain fungi in field, as well as differences in the soluble sugar, fibre and protein content of different cultivars, which can influence fermentation, particularly for example if leguminous crops are grown alongside grasses (Moloney et al., 2021). With the increase in interest in multispecies swards (Moloney et al., 2021), future studies should aim to understand how different mixtures may influence plant-fungal interactions in the soil, the fermentation profile, and mycotoxin concentration of the silage.

3.7. Conclusions

The mycotoxin profile in grass silages of Great Britain include the regulated mycotoxins DON, ZEA and FUM, as well as FUS, PEN, enniatins and ergot alkaloids. Silage fermentation that led to a lower DM, higher lactic acid and higher acetic acid concentration (indicating a slower initial fermentation) was correlated with an increase in mycotoxin content. Drought conditions during growth and sward composition may both impact the mycotoxin content of grass silage, but it is not known whether this was due to in field plant-fungal interactions or subsequent effects on the fermentation during ensiling.

This study was unable to evaluate the effect of aerobic spoilage on mycotoxin concentration of the silage due to a lack of heating across the clamp face, and relatively even compaction. *Penicillium* and *Aspergillus* associated mycotoxins were still present in silage without evidence of aerobic spoilage at opening.

Chapter 4

The relationship between the fermentation profile, aerobic stability and mycotoxin concentration in grass silage: Mini-silo studies

4. The relationship between the fermentation profile, aerobic stability and mycotoxin concentration in grass silage: Mini-silo studies

4.1. Introduction

The study carried out in Chapter 3 identified common mycotoxins found in grass silage and established a positive relationship between lactic and acetic acid concentration and the REQ of total mycotoxin contamination. The study therefore suggested that the fermentation characteristics of a grass silage have a major influence on the resultant mycotoxin content. The study was, however, unable to evaluate the effect of aerobic spoilage on the mycotoxin content of grass silage. Whilst the association between a higher mycotoxin concentration and increased acetic acid in grass silage is in contrast to the literature, which suggests an inhibition of fungal growth by acetic acid (Muck, 2010), mycotoxin concentration is not always correlated with fungal abundance (Manni et al., 2022). It is therefore important to investigate the interaction between grass silage fermentation and aerobic stability on the concentration of mycotoxins.

It has generally been agreed that *Fusarium* and *Claviceps* associated mycotoxin contamination occurs whilst the crop is in the field (Desjardins, 2006; Perincherry et al., 2019), and that *Aspergillus* and *Penicillium* associated mycotoxins appear mainly during the feed out phase (O'Brien, 2010) when the silage is again exposed to oxygen (Uriarte et al., 2001). Nevertheless, the presence of these moulds does not correlate with the concentration of mycotoxins synthesised and it is difficult to determine the precise fungal genus that is responsible for the production of a particular mycotoxin as they are often able to be synthesised by multiple (Cano et al., 2020; Manni et al., 2022). Moreover, *Penicillium* and *Aspergillus* associated mycotoxins were found to contaminate grass silage in Chapter 3, despite no evidence of widespread and extensive aerobic spoilage across the clamps sampled.

The fermentation pathway of a silage can be directly manipulated by the addition of either an obligate homofermentative or facultative heterofermentative (Ho LAB), or an obligate heterofermentative (He LAB) inoculant during harvest (Drouin et al., 2020; Muck, 2010). In addition, the time taken to seal the clamp after initial filling can impact on the speed at which anaerobicity in the clamp can be achieved, affecting the time taken to achieve sufficient acidity to inhibit spoilage organisms such as *Clostridia*, *Enterobacteriaceae* species, yeasts and filamentous fungi (Wróbel et al., 2023). Silage with greater aerobic stability has often been considered advantageous in reducing spoilage organism proliferation during the feed out period and often obligate heterofermentative inoculants have been applied to improve this

specific feature (Ranjit and Kung, 2000). The He LAB pathway yields lactic acid as well as acetic acid, ethanol and propan-1-ol, of which acetic acid and ethanol have been demonstrated to prevent the growth of fungi and yeast during exposure to oxygen at feed out (Oliveira et al., 2017; S. J.W.H. Oude Elferink et al., 2001). However, a solely obligate heterofermentative pathway may also lead to a higher abundance of spoilage organisms that are able to proliferate during the early stages of fermentation, as the pK_a of acetic acid is lower than that of lactic acid, leading to a slower fermentation (McDonald et al., 1991; Uriarte et al., 2001). The obligate heterofermentative fermentation pathway can also result in greater losses of silage DM, and as low silage DM at opening was demonstrated in Chapter 3 as being positively correlated with mycotoxin concentration, this may impact on dairy cow performance (Fink-Gremmels, 2008). It is however currently unclear as to when particular mycotoxins are synthesised during the process of ensiling, from crop harvest to feed out, and it is therefore important to investigate the relationship between silage fermentation characteristics, and subsequent effects of aerobic stability on the mycotoxin concentration of grass silage.

The current chapter details two mini-silo studies. The first study focussed on the controlled manipulation of fermentation with either a Ho LAB or He LAB pathway via the addition of two commercially available silage inoculants, on the fermentation and microbial composition. The second study focussed on the aerobic stability and consequent mycotoxin profile of the silage after exposure to oxygen post-ensiling for 26 days.

4.2. Experiment A: Investigating the relationship between the fermentation profile of grass silage and the presence of mycotoxins.

4.2.1. Hypotheses and objectives

1) The fermentation profile of the two grass silages treated with silage inoculants containing LAB of differing fermentation pathways, would express a different silage profile to that of the control (un-inoculated) and each other.
2) The fermentation pathway of the Ho LAB inoculated grass silage would drop in pH more rapidly than the control or the He LAB inoculated grass silage resulting in a more rapid inhibition of spoilage organisms.
3) Delayed sealing of the silage would impact on all three treatments of their ability to establish a lactic acid bacteria population quickly enough to mitigate the population increase of spoilage organisms.

The objective of this experiment was to investigate any potential relationship between three differently directed fermentations, through the exclusion or inclusion of either a homofermentative (Ho) or heterofermentative (He) based lactic acid bacteria (LAB) inoculant, and either immediate or delayed sealing, on the resultant grass silage profile and mycotoxin content.

4.2.2. Materials and methods

4.2.2.1. Treatments, field and inoculant management

A second cut grass silage of *Lolium perenne* was mown on the 8th August 2023 at Harper Adams University farm (Shropshire, United Kingdom) at 24.4% DM content and harvested via precision chop forage harvester. One tonne of fresh weight (FW) of forage was brought in from a single load and subsampled from to form four equal piles of 200 kg FW (Figure 4-1). These four piles represented replicates 1-4 for each treatment, as detailed in Table 4-1. For each 200 kg FW pile, three 50 kg FW subsamples were taken and spread out onto black plastic sheeting.

Each of the 50 kg FW subsample piles were assigned either Control, He LAB or Ho LAB inoculant treatments. A working solution of each inoculation was made up with tap water as per manufacturer's instruction and applied at a rate of 5 ml/kg FW forage using a 2L pressure spraygun (Hawksmoor, Bauker, Jiangsu, China). For the control treatment piles, tap water was used *in lieu* of inoculant solution and again applied at a rate of 5 ml/kg FW forage.

After application of inoculants, the forage was mixed thoroughly and 1.2 kg FW (± 0.02 kg) packed by hand into 2 L clip top, rubber seal Kilner jars (The Rayware Group, Liverpool, United Kingdom). The process of subsampling, inoculation addition and ensiling was carried out four times in total. Each treatment inoculant was therefore assigned 24 jars, in which 12 were sealed immediately and 12 were left open for 24 hours post ensiling, before being sealed. Timepoint 0 samples of inoculated forage were obtained directly from the piles.

4.2.2.2. Experimental routine

All jars were stored in a controlled environment room at Harper Adams University at 18°C until destructive sampling. At each time point of 2, 5 and 98 d post-ensiling, a total of 24 jars (4 replicates per 6 treatments) each were weighed, emptied, mixed thoroughly, and approximately 700 g of silage subsampled from for the following analyses.

Table 4-1. Grass silage treatments for experiment A, including a silage inoculant treatment, sealing point treatment and timepoint factor for destructive sampling at 0, 2, 5 and 98 d post ensiling.

Silage inoculant	Sealing point (h post ensiling)	Timepoint of destruction (d post ensiling)
Control (no inoculant)	0 h (Immediate)	(0), 2, 5, 98
Control (no inoculant)	24 h (Delayed)	(0), 2, 5, 98
He LAB[1]	0 h (Immediate)	(0), 2, 5, 98
He LAB	24 h (Delayed)	(0), 2, 5, 98
Ho LAB[2]	0 h (Immediate)	(0), 2, 5, 98
Ho LAB	24 h (Delayed)	(0), 2, 5, 98

[1]He LAB: Heterofermentative lactic acid bacteria inoculant.
Lentilactobacillus hilgardii (CNCM I-4785) at 1.00 x10^{11} CFU/g
Lentilactobacillus buchneri (NCIMB 40788 (1k20757)) at 1.00 x10^{11} CFU/g
Pediococcus pentosaceus (NCIMB 12455 (1k2106)) at 5.00 x10^{10} CFU/g
Working concentration: 0.4 g inoculant per L of tap water
Application rate: 5 ml/kg fresh weight forage
[2]Ho LAB: Homofermentative lactic acid bacteria inoculant.
Lactiplantibacillus plantarum (IMI 507026) at 5.00 x10^{5} CFU/g
Pediococcus pentosaceus (IMI 507025) at 5.00 x10^{5} CFU/g
Working concentration: 1 g inoculant per L of tap water
Application rate: 5 ml/kg fresh weight forage

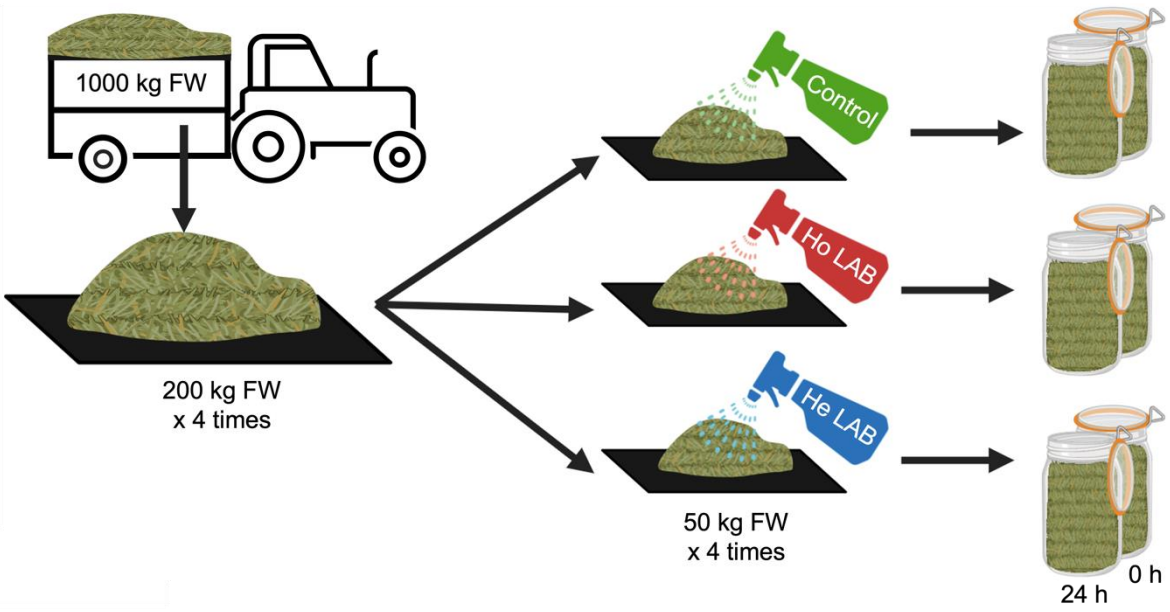


Figure 4-1. Outline of the forage subsampling, forage inoculant application and ensiling of the forage into 2 L Kilner jars, where jars were either sealed immediately (0 h) or sealed 24 h post ensiling (24 h). This subsampling was carried out a total of four times to obtain four replicates for each treatment.

4.2.2.3. Silage fermentation profile analyses

Silage pH measurements for all samples at each time point were recorded as described in Chapter 2. All samples were also subject to DM determination as described in Chapter 2.

4.2.2.4. Silage microbial profile and population counts

Immediately following unpacking, 10 g FW of each subsample was weighed into standard stomacher bags (Seward, West Sussex, United Kingdom) and 90 ml of sterile Ringer's solution was added (ThermoFisher, Massachusetts, United States). Stomacher bags were loaded onto a stomacher (400 Circulator Seward, West Sussex, United Kingdom) for 2 minutes at 230 rpm and kept on ice afterwards, before carrying out subsequent serial dilutions of the supernatant. Five universal tubes of 90 ml of Ringer's solution were autoclaved, and under aseptic conditions, used to obtain serial dilutions of the supernatant ranging from 1.00×10^{-1} ml to 1.00×10^{-6} ml.

For yeast and filamentous fungal counts of each sample, six sterile plates (Sarstedt, Nümbrecht, Germany) pre-prepared with sterile malt extract agar (MEA; Difco™, BD Diagnostics, Berkshire, United Kingdom) supplemented with 10% lactic acid (2.5 ml/L) and

chloramphenicol (34 µg/ml) were inoculated in duplicate with 100 µl of dilutions 1.00 x 10^{-1} – 1.00 x 10^{-3}. Plates were spread evenly with a sterile inoculation spreader (Sarstedt, Nümbrecht, Germany) and stored for 3 d at 23°C in an incubator, before counting. For LAB counts of each sample, twelve sterile plates were inoculated in duplicate with 1000 µl, of dilutions 1.00 x 10^{-1} – 1.00 x 10^{-6} and sterile De Man, Rogosa and Sharpe (MRS; ThermoFisher, Massachusetts, United States) agar supplemented with 1.5 mg / L amphotericin B (ThermoFisher, Massachusetts, United States) poured over and left to set. Plates were stored in an incubator at 30°C for 3 d, before counting. For counts of *Enterobacteriaceae* in each sample, a further twelve sterile plates were inoculated in duplicate with 1000 µl of dilutions 1.00 x 10^{-1} – 1.00 x 10^{-6} and violet-red bile glucose agar (VRBGA; ThermoFisher, Massachusetts, United States) was poured over and left to set. After plates had set, additional VRBGA was poured on top to form an overlay. Plates were stored upside down in an incubator at 37°C for 24 h, before counting.

Colony forming units (CFU) were counted using a manual colony counter (Stuart, Cole-Palmer, Cambridgeshire, United Kingdom), on both duplicates for each dilution where colonies ranged in number from 25-250, and a mean value obtained. Where the total CFU per plate exceeded these numbers, the plate with the subsequent dilution was counted instead, or where this was not possible, plates were sectioned using a Wolffheugel graticule (Sarstedt, Nümbrecht, Germany), CFU within a square sectioned area on the graticule counted and used to estimate total plate CFU. Yeasts and filamentous fungi were distinguished between in CFU counts visually. Smooth, rounded colonies were assigned as yeasts and growths including hyphal structures were assigned as filamentous fungal CFU (Figure 4-2) as described in McGinnis and Tyring, (1996). Additional notes on the colour of fungi present, or yeasts were recorded alongside CFU.

4.2.2.5. Statistical analyses

Data were analysed as a 3 x 2 factorial design using analysis of variance in R (version 4.3.3, R Core Team (2024), Vienna, Austria) to identify the main effects of inoculant treatment and sealing time and any interaction effects, on the measured parameters using the model below:

$$Y_{ij} = \mu + a_i + b_j + (ab)_{ij} + \varepsilon_{ij}$$

Where, Y_{ij}= dependent variable; µ = overall mean; a_i = main effect of the i-th treatment level (Control inoculant, Ho LAB inoculant or He LAB inoculant), b_j = main effect of the j-th sealing time (sealed immediately or after 24 h); $(ab)_{ij}$ = the interaction effect between inoculant treatment (a) and sealing time (b); and ε_{ij}= residual error.

Where appropriate, an additional main effect of timepoint sampled was included in the model, as well as any interaction effect of this factor with others, as described below:

$$Y_{ijk} = \mu + a_i + b_j + c_k + (ab)_{ij} + (ac)_{ik} + (bc)_{jk} + (abc)_{ijk} + \varepsilon_{ijk}$$

Where, Y_{ijk}= dependent variable; μ = overall mean; a_i = main effect of the i-th treatment level (Control inoculant, Ho LAB inoculant or He LAB inoculant), b_j = main effect of the j-th sealing time (sealed immediately or after 24 h); c_k = main effect of k-th timepoint (4 timepoints in experiment A , 6 timepoints in experiment B); $(ab)_{ij}$ = the interaction effect between inoculant treatment (a) and sealing time (b); $(ac)_{ik}$ = the interaction effect between inoculant treatment (a) and timepoint (c); $(bc)_{jk}$ = the interaction effect between sealing time (b) and timepoint (c); $(abc)_{ijk}$ = three-way interaction effect between inoculant treatment (a) and sealing time (b) and timepoint (c); and ε_{ijk}= residual error.

Post-hoc analysis was carried out with Tukey's test in R using the function `HSD.test` in the `agricolae` package and where appropriate, superscripts have been presented to denote differences between means where an interaction effect was considered significant ($p < 0.05$).

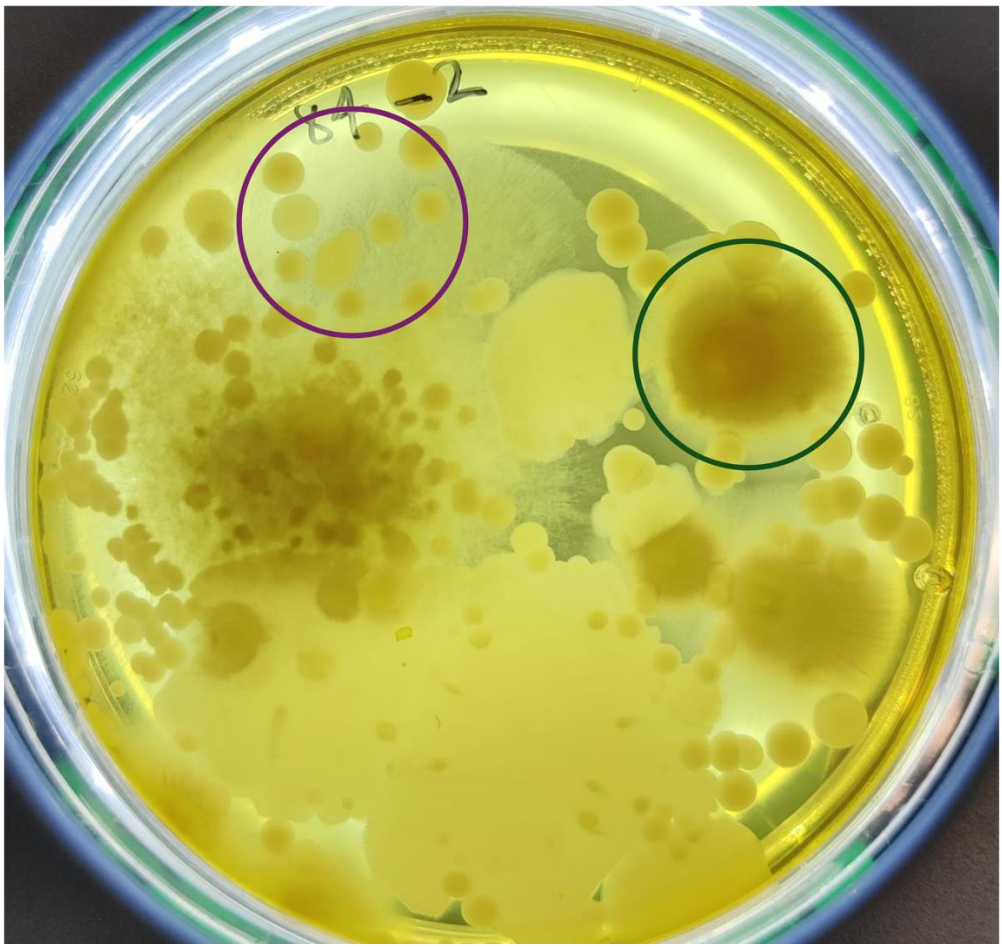


Figure 4-2. Determination of counts of yeast colonies (circled red) and filamentous fungi (circled green) growth on malt extract agar (MEA) plates. Yeast colonies were identified as smooth rounded colonies and filamentous hyphal structures identified as filamentous fungi as described in McGinnis and Tyring, (1996).

4.2.3. Results

4.2.3.1. Silage pH, DM content and DM loss

The mean pH of the grass ensiled at 0 d ranged from 5.35 to 5.44 and decreased across time ($P < 0.001$) to mean pH values of the silage ranging from 3.51 to 4.58 at 98 d post ensiling (Table 4-2). Mean DM content of the silage at 98 d post ensiling ranged from 208 to 234 g/kg, with no effect of inoculant ($P = 0.311$) or sealing time ($P = 0.498$; Table 4-2). Mean DM lost from the silage at 98 d post ensiling ranged from 13.9 – 41.6 g. There was an effect of inoculant ($P = 0.025$), with the lowest mean loss of 14.8 g (± 1.27 g) in the control treated silage, a mean loss of 30.4 g (± 15.9 g) in the Ho LAB treated silage, and the highest mean loss of 32.3 g (± 2.47 g) in the He LAB treated silage (Table 4-2). There was a trend for the effect of sealing time ($P = 0.076$), with a higher mean loss of 30.4 g (± 13.3 g) for silage that had experienced delayed sealing in comparison to a lower mean loss of 21.7 g (± 8.49 g) for silage that was sealed immediately after filling.

Table 4-2. Silage characteristics for each of the grass silage treated with either the Control inoculant (no inoculant), Ho LAB inoculant, or He LAB inoculant, with either immediate sealing (0 h) or delayed sealing (24 h), at time points 0, 2, 5 and 98 d post ensiling.

	Mean silage characteristics							Significance						
	Delayed sealing			Immediate sealing			s.e.d.[1]							
	Con	Ho LAB	He LAB	Con	Ho LAB	He LAB		inoc[2]	seal[3]	time[4]	inoc x time	seal x time	inoc x seal	inoc x seal x time
Silage pH														
0 d	5.44	5.35	5.44	5.44	5.35	5.44	0.193							
2 d	4.06	3.90	3.94	4.04	3.89	3.96	0.115	0.501	0.971	<0.001	0.999	1.000	0.249	0.231
5 d	3.80	3.68	3.77	3.81	3.72	3.71	0.073							
98 d	3.51	4.10	4.27	4.58	3.49	3.81	1.323							
Dry matter content (g/kg)														
98 d	234	208	213	223	232	215	18.5	0.311	0.498	-	-	-	0.158	-
Dry matter lost (g)														
98 d	15.7	41.6	34.0	13.9	19.1	30.5	15.36	0.025	0.076	-	-	-	0.207	-

[1]s.e.d. = standard error of the difference between means, [2]inoc = Effect of inoculant treatment, [3]seal = Effect of sealing time, [4]time = Effect of time (days post ensiling).

4.2.3.2. Silage microbial profile and population counts

There were only effects of time on counts of *Enterobacteriaceae* ($P < 0.001$; Table 4-3).The range of mean counts were between 7.58 and 7.62 log_{10}(CFU/g FW) in the pre-ensiled grass with a combined mean of 7.60 log_{10}(CFU/g FW) compared with between 0.233 and 4.87 log_{10}(CFU/g FW), with a lower combined mean of 2.30 log_{10}(CFU/g FW) at 98 d post ensiling.

Similarly, there were only effects of time ($P < 0.001$; Table 4-3) on mean counts of LAB. The range of mean counts of LAB were between 6.15 and 6.55 log_{10}(CFU/g FW) in the pre-ensiled grass with a combined mean of 6.29 log_{10}(CFU/g FW) compared with between 8.77 and 9.03 log_{10}(CFU/g FW), with a higher combined mean of 8.94 log_{10}(CFU/g FW) at 5 d post ensiling, before decreasing to a mean of 6.38 log_{10}(CFU/g FW) at 98 d post ensiling.

There were effects of sealing time ($P < 0.001$) on the mean counts of filamentous fungi with a range of 2.83 to 6.51 log_{10}(CFU/g FW) and a mean of 4.03 log_{10}(CFU/g FW) for silage that was sealed after 24 hours, in comparison to a lower range of 0.00 – 4.53 log_{10}(CFU/g FW), and a mean of 2.56 log_{10}(CFU/g FW) for silage that was sealed immediately (Table 4-3). There were also effects of time ($P < 0.001$) on the mean counts of filamentous fungi, with a decrease from a mean of 4.52 log_{10}(CFU/g FW) in pre-ensiled grass, compared with a mean of 3.48 log_{10}(CFU/g FW) for silage at 98 d post ensiling.

There was an effect of inoculant ($P = 0.004$) on the mean counts of yeast, with a range of 4.80 – 5.57 log_{10}(CFU/g FW) and a mean of 5.07 log_{10}(CFU/g FW) for silage treated with the control inoculant, a range of 5.08 – 6.42 log_{10}(CFU/g FW) and a higher mean of 5.46 log_{10}(CFU/g FW) for silage treated with the Ho LAB inoculated silage, and a range of 1.97 – 5.99 log_{10}(CFU/g FW) and the lowest mean of 4.69 log_{10}(CFU/g FW) for silage treated with the He LAB inoculated silage (Table 4-3). There was an effect of time ($P < 0.001$) with a mean count of 4.98 log_{10}(CFU/g FW) in pre-ensiled grass compared with a higher mean count of 5.58 log_{10}(CFU/g FW) at 5 d, and a lower mean of 4.46 log_{10}(CFU/g FW) at 98 d post ensiling. There was an interaction between inoculant and time ($P < 0.001$), with silage that was treated with He LAB inoculant only, demonstrating a reduction in mean counts of yeast from 5 d post ensiling, with a mean of 5.86 log_{10}(CFU/g FW), to a mean of 2.53 log_{10}(CFU/g FW) at 98 d post ensiling (Table 4-3).

Table 4-3. Mean log_{10}(CFU/g FW) of *Enterobacteriaceae,* Lactic acid bacteria (LAB), filamentous fungi and yeasts, for each of the grass silage treated with either the Control inoculant (no inoculant), Ho LAB inoculant, or He LAB inoculant, with either immediate sealing (0 h) or delayed sealing (24 h), at time points 0, 2, 5 and 98 d post ensiling.

	Mean log_{10}(CFU/g FW)						s.e.d.[1]	Significance						
	Delayed sealing			Immediate sealing										
	Con	Ho LAB	He LAB	Con	Ho LAB	He LAB		inoc[2]	seal[3]	time[4]	inoc x time	seal x time	inoc x seal	inoc x seal x time
Enterobacteriaceae														
0 d	7.60	7.58	7.62	7.60	7.58	7.62	0.422	0.195	0.070	<0.001	0.262	0.529	0.440	0.066
2 d	6.88	5.77	5.58	5.97	4.86	5.75	1.552							
5 d	0.000	1.80	4.39	0.675	0.886	1.04	2.9236							
98 d	2.13	4.87	2.14	1.31	0.233	3.14	3.0382							
Lactic acid bacteria														
0 d	6.17	6.55	6.15	6.17	6.55	6.15	0.886	0.141	0.942	<0.001	0.457	0.707	0.767	0.994
2 d	9.18	9.10	8.81	9.12	9.14	9.12	0.521							
5 d	9.00	8.99	8.95	8.89	8.77	9.03	0.183							
98 d	6.71	6.44	6.36	6.58	6.07	6.10	0.739							
Filamentous fungi														
0 d	4.43	4.53	4.61	4.43	4.53	4.61	0.390	0.500	<0.001	<0.001	0.560	0.175	0.514	0.449
2 d	2.88	3.37	2.83	1.90	0.000	1.40	2.6343							
5 d	3.58	3.81	4.91	1.42	2.86	2.11	1.951							
98 d	3.53	6.51	3.35	1.73	2.56	3.21	3.247							
Yeast														
0 d	4.80^{ab}	5.08^{ab}	5.05^{ab}	4.80^{ab}	5.08^{ab}	5.05^{ab}	0.521	0.004	0.105	0.001	<0.001	0.439	0.879	0.768
2 d	5.57^{ab}	5.21^{ab}	5.45^{ab}	4.86^{ab}	5.36^{ab}	5.19^{ab}	0.984							
5 d	5.48^{ab}	5.50^{ab}	5.99^{a}	4.95^{ab}	5.81^{a}	5.72^{a}	0.769							
98 d	5.09^{ab}	6.42^{a}	3.08^{bc}	4.97^{ab}	5.22^{ab}	1.97^{c}	1.779							

[1]s.e.d. = standard error of the difference between means, [2]inoc = Effect of inoculant treatment, [3]seal = Effect of sealing time, [4]time = Effect of time (days post ensiling), [a-d] Superscripts denote values across rows and columns that are significantly different with Tukey's test.

4.3. Experiment B: Investigating the relationship between aerobic spoilage of grass silage on the presence of mycotoxins.

4.3.1. Hypotheses and objectives

1) The fermentation profile of the two grass silage treated with silage inoculants containing LAB of differing fermentation pathways, would express different rates of aerobic spoilage.
2) The higher acetic acid yield of the He LAB inoculated grass silage would mean that aerobic spoilage would occur less rapidly than for the control or the Ho LAB inoculated grass silage, yet mycotoxin concentration may be increased, due to the association with acetic acid concentration.
3) Delayed sealing of the silage would impact on all three treatments, leading to more rapid aerobic spoilage than in silage that was sealed immediately.

The objective of this experiment was to investigate any potential relationship between three differently directed fermentations, through the exclusion or inclusion of either a homofermentative (Ho) or heterofermentative (He) based lactic acid bacteria (LAB) inoculant, and either immediate or delayed sealing, on the aerobic spoilage and subsequent mycotoxin content of grass silage.

4.3.2. Materials and methods

4.3.2.1. Treatments and study design

At the same time as the jars were ensiled in Section 4.2.2.2, a total of 24, 30 litre clip top plastic barrels (CJK Packaging, Derbyshire, United Kingdom) were ensiled with approximately 17 kg fresh weight grass from the three piles treated with either control (no inoculant), a homofermentative additive or a heterofermentative additive. Barrels were also either sealed immediately after filling or left open for 24 hours before sealing, as with the jars in experiment A. Barrels were then left for 103 days in a controlled environment room at 18 °C.

One hundred and three days after ensiling, barrel lids were opened, barrels weighed, and approximately 8 kg fresh weight from each barrel was emptied onto a plastic sheet before being mixed thoroughly. The mixed sample was subsampled for wet chemical analysis of VFA content and alcohols as in Chapter 2 and water soluble carbohydrates, microbial counts as in Section 4.2.2.4, pH as described in Chapter 2, 200 g FW for mycotoxin analysis as described in Chapter 2, and finally an archive sample of approximately 350 g FW.

The following evaluation of the aerobic stability of the silage was based on the methods described by Honig, (1986). Approximately 350 g fresh weight (± 2.00 g) subsamples of the mixed silage were placed into five 1 L lever lid paint tins (Spray Guns Direct Ltd., Derbyshire, United Kingdom) per barrel (5 tins per 24 barrels, 120 tins in total). Paint tins had a 10 mm dia hole drilled into the bottom at the centre prior to filling. Approximately half of the 350 g fresh weight of mixed silage was placed in the tin first, before a temperature recording device or "*Thermocron"* (1-Wire Thermocron iButton®, Measurement Systems Ltd., Berkshire, United Kingdom) was placed in the centre and then the remaining silage was placed on top. Thermocrons were programmed using the 1-Wire software provided by the manufacturer, to record the temperature of the immediate environment every 15 minutes. A 20 x 20 cm cut square of 8 micron thick polyethylene plastic was placed over the top of each tin, pulled taught and secured with an elastic band and a 10 mm dia hole was pierced into the centre of the plastic sheet square. Three thermocrons were placed inside the temperature controlled room at different locations in order to obtain a reference for the ambient temperature.

The packed and sealed tins were then weighed. Polystyrene boxes (internal dimensions: L: 14 cm, H: 14 cm, W: 14 cm and 7 cm wall thickness; MP001, Longvalley Packaging, Essex, United Kingdom), with lids, were set upside down, so that the top of the lids of the boxes were on the floor. Tins were placed inside boxes and each placed on three M10 hex nuts (Screwfix, Somerset, United Kingdom) on top of the box lids with the box placed lightly over the top as to not seal the boxes shut. The nuts raised the tins by 12.5 mm from the lid, to allow for air circulation through and into the bottom hole of the tin. Tins inside boxes were left in a temperature controlled room at 18 °C until destructive harvest at either 2, 4, 6, 16 or 26 days of aerobic exposure.

Table 4-4. Grass silage treatments for experiment B, including a silage inoculant treatment, sealing point treatment and timepoint factor for destructive sampling at 0, 2, 4, 8, 16 and 26 d of aerobic exposure.

Silage inoculant	Sealing point (h post ensiling)	Timepoint of destruction (d of aerobic exposure)
Control (no inoculant)	0 h (Immediate)	(0), 2, 4, 8, 16, 26
Control (no inoculant)	24 h (Delayed)	(0), 2, 4, 8, 16, 26
He LAB[1]	0 h (Immediate)	(0), 2, 4, 8, 16, 26
He LAB	24 h (Delayed)	(0), 2, 4, 8, 16, 26
Ho LAB[2]	0 h (Immediate)	(0), 2, 4, 8, 16, 26
Ho LAB	24 h (Delayed)	(0), 2, 4, 8, 16, 26

[1]He LAB: Heterofermentative lactic acid bacteria inoculant.
Lentilactobacillus hilgardii (CNCM I-4785) at 1.00 $x10^{11}$ CFU/g
Lentilactobacillus buchneri (NCIMB 40788 (1k20757)) at 1.00 $x10^{11}$ CFU/g
Pediococcus pentosaceus (NCIMB 12455 (1k2106)) at 5.00 $x10^{10}$ CFU/g
Working concentration: 0.4 g inoculant per L of tap water
Application rate: 5 ml/kg fresh weight forage
[2]Ho LAB: Homofermentative lactic acid bacteria inoculant.
Lactiplantibacillus plantarum (IMI 507026) at 5.00 $x10^{5}$ CFU/g
Pediococcus pentosaceus (IMI 507025) at 5.00 $x10^{5}$ CFU/g
Working concentration: 1 g inoculant per L of tap water
Application rate: 5 ml/kg fresh weight forage

4.3.3. Experimental routine

At each time point of 2, 4, 8, 16, and 26 d of aerobic exposure, tins were removed from the polystyrene boxes, weighed, completely emptied into a plastic bowl, and mixed thoroughly. Ten grams of fresh weight of mixed silage was placed into a standard stomacher bag (Seward, West Sussex, United Kingdom) for enumeration of yeast and filamentous fungal counts, and another stomacher bag was filled with 10 g FW for pH determination as described in Chapter 2. The remaining silage was placed into an archive sample bag and frozen at -20 °C. Silage of approximately 100 g fresh weight from tins at 8 d of aerobic exposure were put in an additional sample bag and were sent for mycotoxin analysis, as described in Chapter 2.

Thermocrons were retrieved from the tins and the recorded temperatures were accessed using the 1-Wire software provided by the manufacturer. As described by Honig, (1986) the time taken for temperatures recorded inside the tins to reach 2 degrees above ambient temperature were used for subsequent analysis.

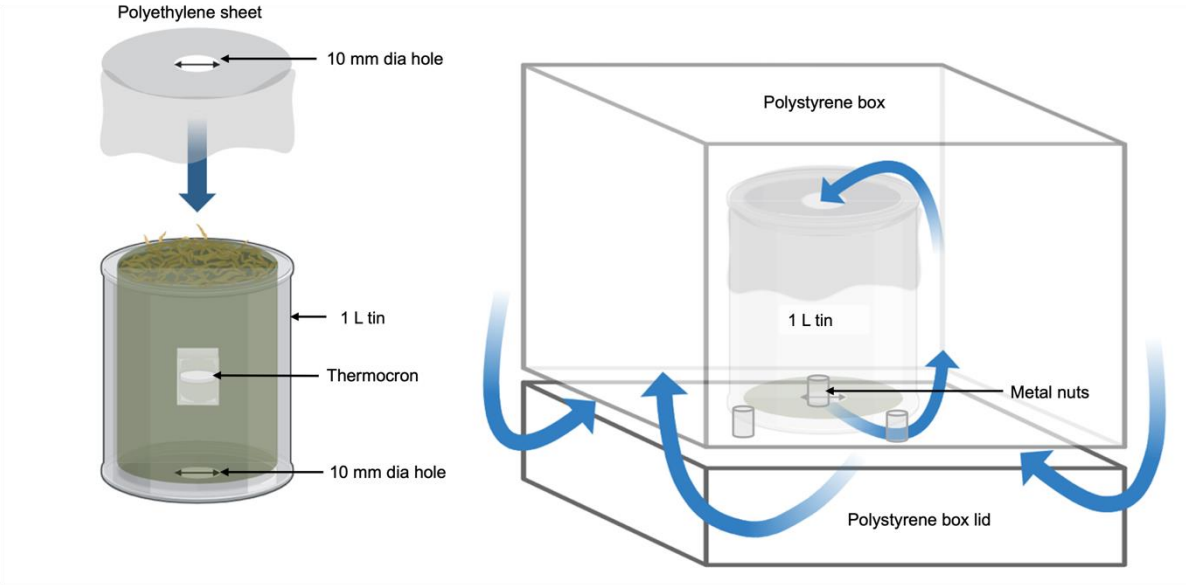


Figure 4-3. Aluminium tins with a 10 mm dia hole drilled in the bottom, were filled with approximately 350 g fresh weight of silage, with a temperature recording device (Thermocron) placed in the centre. Tins were sealed with a polyethylene sheet and secured with elastic band before an additional 10 mm dia was pierced into the plastic sheet. Tins were then placed inside an upside down polystyrene box, where air could circulate and enter and exit the tins through the top or bottom 10 mm dia holes. The method was based on Honig, (1986).

4.3.4. Results

4.3.4.1. Silage fermentation profile analyses of barrels opened after 103 days of ensiling

In the barrels that were opened after 103 days of ensiling, there was an effect of inoculant ($P = 0.010$; Table 4-5) on the mean DM content of the silage, with the highest means in silage that were treated with the control inoculant (216 g/kg, ±1.41), or Ho LAB inoculant (215 g/kg, ± 3.54) in comparison to the lowest in silage that were treated with the He LAB inoculant (207 g/kg ± 1.41). The mean DM lost from silage in barrels after 103 d of ensiling ranged from 32.2 – 41 g of DM.

There was an effect of inoculant on mean pH ($P < 0.001$) where silage treated with He LAB inoculant demonstrated a higher mean pH of 3.68 ± 0.007, in comparison to silage treated with control or Ho LAB inoculants, with means of 3.52 ± 0.007 and 3.51 ± 0.007, respectively.

Silage treated with He LAB inoculant ($P < 0.001$), demonstrated the lowest mean water soluble carbohydrate (WSC) concentration of 6.49 g/kg DM, ± 0.467, in comparison to mean WSC for

control and Ho LAB inoculant treated silage with means of 18.3 g/kg DM, ± 3.61, and 16.5 g/kg DM, ± 2.05, respectively.

Similarly, silage treated with He LAB inoculant ($P < 0.001$), demonstrated the lowest lactic acid concentration of 75.6 g/kg DM, ± 1.63, in comparison to control and Ho LAB inoculant treated silage, with means of 102 g/kg DM, ± 0.707 and 104 g/kg DM, ± 2.83, respectively. The opposite was demonstrated for acetic acid concentration where silage treated with He LAB inoculant ($P < 0.001$) had the highest mean concentration of 38.1 g/kg DM, ± 0.778, in comparison to control and Ho LAB inoculant treated silage, with means of 18.2 g/kg DM, ± 0.424 and 14.5 g/kg DM, ±. 0.212, respectively.

There was an effect of inoculant on concentrations of propionic ($P < 0.001$) and butyric acid ($P = 0.004$), where silage treated with the He LAB inoculant, demonstrated the highest mean concentrations of both propionic (0.229 g/kg DM, ± 0.1390) and butyric acids (0.141 g/kg DM, ± 0.0523) in comparison to the means for the control (0.047 g/kg DM, ± 0.063, and 0.00 g/kg, respectively) and Ho LAB inoculant treated silage (0.008 g/kg DM, ± 0.0113, and 0.0375 g/kg DM ± 0.0403 , respectively). There was an interaction between inoculant and sealing time for mean propionic acid concentration ($P = 0.017$), with the highest mean for He LAB inoculant treated silage, when sealing was delayed (0.327 g/kg DM), in comparison to control and Ho LAB inoculant treated silage (0.009 g/kg DM, ± 0.010), but this difference was not found when He LAB inoculant treated silage were sealed immediately (0.131 g/kg DM) in comparison to control and Ho LAB inoculant treated silage (0.091 g/kg DM, ± 0.0643).

There was an effect of inoculant on concentrations of valeric ($P < 0.009$) and isovaleric acid ($P = 0.008$), where silage treated with the He LAB inoculant, demonstrated the lowest mean concentrations of both valeric (0.121 g/kg DM, ± 0.0014) and isovaleric acids (0.00 g/kg DM) in comparison to the means for the control (0.116 g/kg DM, ± 0.0007, and 0.017 g/kg, ± 0.0240 respectively) and Ho LAB inoculant treated silage (0.117 g/kg DM, ± 0.0021, and 0.004 g/kg DM ± 0.0049, respectively). There was an interaction between inoculant and sealing time for mean isovaleric acid concentration ($P = 0.002$), with the highest mean for control inoculant treated silage, when sealing was immediate (0.034 g/kg DM), in comparison to He LAB and Ho LAB inoculant treated silage (0.00 g/kg DM), but this difference was not found when control inoculant treated silage were sealed after 24 hours (0.00 g/kg DM) in comparison to control and Ho LAB inoculant treated silage (0.007 g/kg DM, ± 0.0049).

There was an effect of inoculant ($P = 0.003$) on mean ethanol concentration where Ho LAB inoculant treated silage had the highest mean ethanol concentration of 14.5 g/kg DM, ± 1.41, in comparison to control inoculant treated silage with a mean of 10.9 g/kg DM, ± 0.778, and a

mean of 8.50 g/kg DM, ± 1.90, for He LAB inoculant treated silage. There was also an effect of inoculant on mean propan-1-ol ($P < 0.001$) and propane-1,2-diol ($P < 0.001$) concentration, where silage treated with He LAB inoculant, demonstrated the highest mean for both propan-1-ol (0.558 g/kg DM, ± 0.016) and for propane-1,2-diol (25.8 g/kg DM, ± 1.56) in comparison to Ho LAB inoculant treated silage with means of 0.033 g/kg, ± 0.0014 and 0.0074 g/kg DM, ± 0.00494 respectively, and means of 0.117 g/kg DM, ± 0.0177, and 0.264 g/kg DM, ± 0.0368 respectively, for the control inoculated silage.

Total VFAs ranged from 113 – 121 g/kg DM. There was an effect of inoculant on lactic acid to acetic acid ratio ($P < 0.001$), lactic acid to total VFAs ($P < 0.001$) and also lactic acid to total VFAs and alcohols ($P < 0.001$). Mean lactic acid to acetic acid ratio was highest in silage treated with Ho LAB inoculant (7.27 g/kg DM, ± 0.099), second highest being the silage treated with the control inoculant (5.56 g/kg DM, ± 0.156) and He LAB inoculant treated silage demonstrating the lowest mean (2.10 g/kg DM, ± 0.120). Consequently, the mean ratio of lactic acid to total VFA for Ho LAB inoculant treated silage was again highest (7.20 g/kg DM, ± 0.106) in comparison to means for control (5.51 g/kg DM, ± 0.184) and He LAB inoculant treated silage (2.10 g/kg DM, ± 0.120). Similarly, the mean ratio of lactic acid to total VFAs and alcohol for Ho LAB inoculant treated silage was highest (3.62 g/kg DM, ± 0.191) in comparison to means for control (3.51 g/kg DM, ± 0.042) and He LAB inoculant treated silage (1.12 g/kg DM, ± 0.057)

Table 4-5. Mean silage characteristics for each grass silage treated with either the Control inoculant (no inoculant), Ho LAB inoculant, or He LAB inoculant, with either immediate sealing (0 h) or delayed sealing (24 h), within barrels, at 103 d post ensiling.

Barrels	Mean silage characteristics						s.e.d.[1]	Significance		
	Delayed sealing			Immediate sealing						
	Con	Ho LAB	He LAB	Con	Ho LAB	He LAB		inoc[2]	seal[3]	inoc x seal
Dry matter (g/kg)	215	212	206	217	217	208	7.01	0.010	0.196	0.782
Dry matter lost (g)	34.7	37.7	41.0	32.8	32.2	39.0	8.51	0.189	0.283	0.845
pH	3.51	3.51	3.68	3.52	3.50	3.67	0.079	< 0.001	0.852	0.954
Water soluble carbohydrates (g/kg DM)	20.8	17.9	6.82	15.7	15.0	6.16	5.290	< 0.001	0.119	0.601
Lactic acid (g/kg DM)	102	106	76.7	101	102	74.4	10.93	< 0.001	0.487	0.944
Acetic acid (g/kg DM)	17.9	14.6	37.5	18.5	14.3	38.6	5.64	< 0.001	0.825	0.953
Propionic acid (g/kg DM)	0.002[b]	0.016[b]	0.327[a]	0.091[b]	0.000[b]	0.131[ab]	0.1101	< 0.001	0.277	0.017
Butyric acid (g/kg DM)	0.000	0.009	0.104	0.000	0.066	0.178	0.0930	0.004	0.177	0.604
Isobutyric acid (g/kg DM)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-	-	-
Valeric acid (g/kg DM)	0.116	0.118	0.122	0.115	0.115	0.120	0.0039	0.009	0.209	0.827
Isovaleric acid (g/kg DM)	0.000[b]	0.007[b]	0.000[b]	0.034[a]	0.000[b]	0.000[b]	0.0122	0.008	0.040	0.002
Hexanoic acid (g/kg DM)	0.000	0.000	0.000	0.022	0.000	0.000	0.0138	0.113	0.134	0.113
Heptanoic acid (g/kg DM)	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-	-	-
Ethanol (g/kg DM)	10.3	13.5	9.84	11.4	15.5	7.16	3.627	0.003	0.901	0.265
Propan-1-ol (g/kg DM)	0.104	0.033	0.569	0.129	0.031	0.547	0.0976	< 0.001	0.995	0.843
Propane-1,2-diol (g/kg DM)	0.238	0.011	24.7	0.290	0.004	26.9	7.45	< 0.001	0.772	0.922
Total VFAs (g/kg DM)	120	121	115	119	116	113	6.54	0.114	0.336	0.735
Lactic acid : Acetic acid	5.67	7.34	2.21	5.45	7.20	2.03	0.902	< 0.001	0.554	0.993
Lactic acid : Total VFAs	5.64	7.27	2.18	5.38	7.12	2.01	0.903	< 0.001	0.529	0.988
Lactic acid : Total VFAs + Alcohols	3.54	3.75	1.16	3.29	3.48	1.08	0.492	< 0.001	0.239	0.878

[1]s.e.d. = standard error of the difference between means, [2]inoc = Effect of inoculant treatment, [3]seal = Effect of sealing time, [a-c] Superscripts denote values across rows that are significantly different with Tukey's test.

4.3.4.2. Microbial profile of silage in barrels after 103 days of ensiling

At 103 d post ensiling for silage in barrels, there was no effect of inoculant (P = 0.449), sealing time (P = 0.210) or an interaction (P = 0.638; Table 4-6) on mean counts of *Enterobacteriaceae* with a mean of 1.88 log_{10}(CFU/g FW^{-1}).

There was an effect of inoculant on mean lactic acid bacteria counts with the highest mean of 8.86 log_{10}(CFU/g FW^{-1}) in silage treated with He LAB inoculant, a mean of 7.05 log_{10}(CFU/g FW^{-1}) for control inoculant treated silage, and a mean of 6.16 log_{10}(CFU/g FW^{-1}) for Ho LAB inoculant treated silage (Table 4-6). There was also an effect of sealing time (P = 0.020; Table 4-6) on the mean counts of lactic bacteria count, with the highest mean of 7.48 log_{10}(CFU/g FW^{-1}) for silage sealed immediately, in comparison to a mean of 7.38 log_{10}(CFU/g FW^{-1}) for silage that experienced a delayed sealing. There was no effect of an interaction between inoculant and sealing time on the mean lactic acid bacteria counts (P = 0.435; Table 4-6).

There was no effect of inoculant (P = 0.116), sealing time (P = 0.160) or interaction (P = 0.571) for mean counts of filamentous fungi, with a mean of 1.79 log_{10}(CFU/g FW^{-1}); Table 4-6). There was an effect of inoculant on the mean counts of yeast with the highest mean of 5.51 log_{10}(CFU/g FW^{-1}) in silage that were treated with Ho LAB inoculant, followed by a mean of 5.06 log_{10}(CFU/g FW^{-1}) for silage treated with the control inoculant, and the lowest mean of 1.80 log_{10}(CFU/g FW^{-1}) for silage treated with the He LAB inoculant. There was no effect of sealing time (P = 0.150) on mean yeast counts, but there was an interaction (P = 0.016), where silage treated with He LAB inoculant demonstrated a significantly lower mean when sealed immediately (0.825 log_{10}(CFU/g FW^{-1})) in comparison to when sealing was delayed (2.77 log_{10}(CFU/g FW^{-1})).

4.3.4.3. Microbial profile of silage after 2, 4, 8, 16 or 26 days of aerobic exposure

There was an effect of inoculant (P = 0.001) on mean filamentous fungi counts with the lowest mean (5.8 log_{10}(CFU/g FW^{-1})) demonstrated by silage treated with He LAB inoculant, followed by the Ho LAB inoculant treated silage (6.51 log_{10}(CFU/g FW^{-1})) and the highest mean for silage treated with Ho LAB inoculant (6.91 log_{10}(CFU/g FW^{-1}); Table 4-7). There was an effect of time (P < 0.001) with an increase from a mean of 2.86 log_{10}(CFU/g FW^{-1}) to a mean of 7.89 log_{10}(CFU/g FW^{-1}) from 2 d of aerobic exposure to 26 d of aerobic exposure for fungal counts. There was also an interaction between inoculant and sealing time (P = 0.005) where for silage that experienced delayed sealing, a mean of 6.59 log_{10}(CFU/g FW^{-1}) was demonstrated for control inoculant treated silage, a mean of 6.37 log_{10}(CFU/g FW^{-1}) for Ho LAB inoculant treated silage, and a mean of 6.46 log_{10}(CFU/g FW^{-1}) for He LAB inoculant treated silage. However,

for silage that was sealed immediately after filling, the highest mean of 7.23 log_{10}(CFU/g FW^{-1}) was demonstrated for control inoculant treated silage, a mean of 6.65 log_{10}(CFU/g FW^{-1}) for Ho LAB inoculant treated silage, and the lowest mean of 5.15 log_{10}(CFU/g FW^{-1}) for He LAB inoculant treated silage.

There was an effect of inoculant ($P < 0.001$) on mean yeast counts, where silage treated with He LAB inoculant demonstrated the lowest mean of 4.87 log_{10}(CFU/g FW^{-1}), in comparison to silage treated with control and Ho LAB inoculants, with means of 8.00 and 8.04 log_{10}(CFU/g FW^{-1}), respectively (Table 4-7). There was also an effect of sealing time on the mean yeast counts ($P = 0.002$), with a higher mean demonstrated for silage that experienced delayed sealing of 7.29 log_{10}(CFU/g FW^{-1}), and a lower mean of 6.64 log_{10}(CFU/g FW^{-1}) demonstrated for silage that was sealed immediately after filling. There was also an effect of time ($P < 0.001$) on mean yeast counts, with a general increase across the time points; a mean of 5.52 log_{10}(CFU/g FW^{-1}) at 2 d of aerobic exposure, a mean of 6.96 log_{10}(CFU/g FW^{-1}) at 4 d of aerobic exposure, a mean of 7.29 log_{10}(CFU/g FW^{-1}) at 8 d of aerobic exposure, rising to the highest mean at 16 d of aerobic exposure of 7.74 log_{10}(CFU/g FW^{-1}), and finally a mean of 7.33 log_{10}(CFU/g FW^{-1}) at 26 d of aerobic exposure.

There was an interaction between inoculant and time for mean yeast counts ($P = 0.050$), where control inoculant treated silage ranged from 6.76 – 8.69 log_{10}(CFU/g FW^{-1}) across the 5 timepoints, Ho LAB treated silage ranged from 7.28 – 8.62 log_{10}(CFU/g FW^{-1}) across the 5 timepoints, and the lowest mean range of 2.54 - 5.93 log_{10}(CFU/g FW^{-1}) demonstrated by He LAB inoculant treated silage across the 5 timepoints. There was also an interaction between inoculant and sealing time ($P = 0.005$), where for silage that experienced delayed sealing, the highest mean of 8.19 log_{10}(CFU/g FW^{-1}) was demonstrated by Ho LAB inoculant treated silage, a mean of 8.03 log_{10}(CFU/g FW^{-1}), for control inoculant treated silage, and the lowest mean of 5.67 log_{10}(CFU/g FW^{-1}), for He LAB inoculant treated silage. In silage that was sealed immediately after filling, the mean for control inoculant treated silage was 7.97 log_{10}(CFU/g FW^{-1}), the mean for Ho LAB inoculant treated silage was 7.90 log_{10}(CFU/g FW^{-1}), and a lower mean of 4.06 log_{10}(CFU/g FW^{-1}), was demonstrated by He LAB inoculant treated silage.

Table 4-6. Mean log_{10}(CFU/g FW^{-1}) of *Enterobacteriaceae,* LAB, filamentous fungi and yeasts, for each grass silage treated with either the Control inoculant (no inoculant), Ho LAB inoculant, or He LAB inoculant, with either immediate sealing (0 h) or delayed sealing (24 h), within barrels, at 103 d post ensiling.

Barrels at 103 d post ensiling	Mean log_{10}(CFU/g FW^{-1})						s.e.d.	Significance		
	Delayed sealing			Immediate sealing						
	Con	Ho LAB	He LAB	Con	Ho LAB	He LAB		inoc	seal	inoc x seal
Enterobacteriaceae	1.79	1.41	1.60	2.73	2.17	1.58	1.316	0.449	0.210	0.638
Lactic acid bacteria	6.93	6.11	8.65	7.16	6.21	9.06	0.295	< 0.001	0.020	0.435
Filamentous fungi	2.76	1.82	1.82	2.82	0.500	1.04	1.9000	0.116	0.160	0.571
Yeast	4.91^{a}	5.44^{a}	2.77^{b}	5.21^{a}	5.58^{a}	0.825^{c}	1.0200	< 0.001	0.150	0.016

$^{a-b}$ Superscripts denote values across rows that are significantly different with Tukey's test.

Table 4-7. Mean log_{10}(CFU/g FW^{-1}) of filamentous fungi and yeasts for each grass silage treated with either the Control inoculant (no inoculant), Ho LAB inoculant, or He LAB inoculant, with either immediate sealing (0 h) or delayed sealing (24 h) after 2, 4, 8, 16 or 26 d of aerobic exposure.

Tins	Mean log_{10}(CFU/g FW^{-1})						s.e.d.	Significance						
	Delayed sealing			Immediate sealing										
	Con	Ho LAB	He LAB	Con	Ho LAB	He LAB		inoc	seal	time	inoc x time	seal x time	inoc x seal	inoc x seal x time
Filamentous fungi														
2 d	2.96^{ef}	2.67^{ef}	3.52^{def}	4.29^{cdef}	2.56^{ef}	1.18^{f}	2.651							
4 d	6.03^{abcde}	5.54^{abcde}	5.67^{abcde}	7.27^{abc}	7.60^{abc}	4.70^{bcdef}	3.493							
8 d	7.70^{abc}	7.75^{abc}	7.04^{abcd}	8.07^{ab}	7.61^{abc}	4.81^{abcdef}	1.455	0.001	0.694	< 0.001	0.591	0.450	0.005	0.708
16 d	8.18^{ab}	8.08^{ab}	8.02^{ab}	8.43^{a}	7.86^{abc}	7.41^{abc}	0.647							
26 d	8.09^{ab}	7.79^{abc}	8.06^{ab}	8.11^{ab}	7.64^{abc}	7.63^{abc}	0.630							
Yeast														
2 d	6.60^{abcdefg}	7.37^{abcde}	3.30^{hi}	6.91^{abcdef}	7.19^{abcdef}	1.77^{i}	1.666							
4 d	8.27^{abcd}	8.15^{abcd}	5.36^{defgh}	7.98^{abcde}	8.41^{abc}	3.60^{ghi}	1.432							
8 d	8.28^{abcd}	8.39^{abc}	7.23^{abcdef}	7.93^{abcde}	7.76^{abcde}	4.14^{fghi}	2.344	< 0.001	0.002	< 0.001	0.050	0.526	0.005	0.746
16 d	8.66^{ab}	8.74^{a}	6.69^{abcdef}	8.71^{ab}	8.50^{abc}	5.16^{efgh}	1.804							
26 d	8.34^{abc}	8.29^{abcd}	5.75^{bcdefgh}	8.34^{abc}	7.62^{abcde}	5.65^{cdefgh}	0.548							

$^{a-i}$ Superscripts denote values across rows and columns that are significantly different with Tukey's test.

4.3.4.4. DM loss after experiencing 0, 2, 4, 8, 16 or 26 days of aerobic exposure

There was an effect of inoculant ($P < 0.001$) on DM lost, with a mean of 420 g lost from silage treated with the control inoculant, a mean of 438 g lost from silage treated with the Ho LAB inoculant, and a lower mean of 260 g lost from the He LAB inoculant treated silage. There was an effect of sealing time ($P = 0.031$) on DM lost also, with the highest mean of 391 g demonstrated by silage that experienced delayed sealing, in comparison to a mean of 354 g in silage that was sealed immediately after filling. There was an effect of time ($P < 0.001$) with means of 32.8 g at 2 d, 157 at 4 d, 284 g at 8 d, 560 g at 16 d, and finally a mean of 831 g at 26 d of aerobic exposure.

There was an interaction between inoculant and time ($P = 0.011$), where the mean DM loss for the control inoculant treated grass silage ranged from 28.8 to 871 g across the five time points, a range of 43.8 – 927 g across the five time points for the Ho LAB inoculant treated grass silage and a range of 25.8 – 697 g across the five time points for the He LAB inoculant treated grass silage. An interaction between inoculant and sealing time ($P = 0.027$) was also demonstrated where there was a larger difference between mean DM loss in grass silage that was treated with He LAB that either experienced delayed sealing (311 g) in comparison to immediate sealing (209 g). Grass silage treated with the control inoculant demonstrated a mean DM loss of 424 g and 425 g with respect to a delayed sealing or an immediate sealing respectively, and a mean DM loss of 439 g and 438 g in grass silage treated with Ho LAB inoculant and either a delayed sealing or an immediate sealing, respectively.

4.3.4.5. Grass silage pH after experiencing 0, 2, 4, 8, 16 or 26 days of aerobic exposure

There was an effect of inoculant ($P < 0.001$) on pH, with a mean of 6.90 in silage treated with the control inoculant, a higher mean of 6.92 in silage treated with the Ho LAB inoculant, and the lowest mean pH of 5.65 in the He LAB inoculant treated silage. There was an effect of sealing time ($P = 0.043$) on mean pH values also, with the highest mean of 6.70 demonstrated by silage that experienced delayed sealing, in comparison to a mean of 6.29 in silage that was sealed immediately after filling. There was an effect of time ($P < 0.001$) with mean pH values of 3.63 at 2 d, 6.11 at 4 d, 6.84 g at 8 d, 7.69 g at 16 d, and finally a mean of 8.19 g at 26 d of aerobic exposure.

There was an interaction between inoculant and time ($P < 0.001$), where the mean pH values for the control inoculant treated grass silage ranged from 3.60 to 8.17 across the five time points, a range of 3.54 – 8.26 across the five time points for the Ho LAB inoculant treated

grass silage and a range of 3.77 – 8.15 across the five time points for the He LAB inoculant treated grass silage. The He LAB inoculant treated grass silage however, at 4 and 8 d of aerobic exposure maintained a mean pH value of approximately 2.5 units lower than that of the control and Ho LAB inoculant treated grass silage at the two time points. By 16 d of aerobic exposure, this difference had decreased to approximately 1 pH unit, and even further to between 0.02 and 0.11 pH units at 26 d of aerobic exposure in comparison to the control and Ho LAB inoculant treated grass silage, respectively. There was a trend for an interaction between inoculant and sealing time (P = 0.059)

4.3.4.6. Hours taken for grass silage to reach 2 °C above ambient temperature

There was an effect of inoculant (P < 0.001) on the number of hours required for silage to reach 2 °C above ambient temperature, with a mean of 55.8 h for silage treated with the control inoculant, a lower mean of 52.9 h for silage treated with the Ho LAB inoculant, and the highest mean of 160 h in the He LAB inoculant treated silage.

There was an interaction between inoculant and time (P < 0.001) where the mean hours taken for grass silage to reach 2 °C above ambient temperature for the control inoculant treated grass silage ranged from 41.3 to 66.5 h across the five time points, a range of 43.4 to 59.1 h across the five time points for the Ho LAB inoculant treated grass silage and a range of 72.2 to 215 h across the five time points for the He LAB inoculant treated grass silage.

4.3.4.7. Maximum temperature reached in the grass silage

There was an effect of inoculant (P < 0.001) on the maximum temperature reached in silage with a mean of 27.3 °C in silage treated with the control inoculant, a mean of 27.7 °C for silage treated with the Ho LAB inoculant, and the lowest mean of 22.3 °C in the He LAB inoculant treated silage. There was also an effect of sealing time (P = 0.005) where mean maximum temperature reached was higher in grass silage that experienced delayed sealing (26.4 °C) in comparison to grass silage that experienced immediate sealing (25.1 °C). There was an effect of time (P < 0.001) with mean maximum temperature values of 18.8 °C at 2 d, 26.6 °C at 4 d, 27.1 °C at 8 d, 28.2 °C at 16 d, and finally a mean of 28.0 °C at 26 d of aerobic exposure.

There was an interaction between inoculant and time (P < 0.001) on the maximum temperature reached in silage across the five time points, with a range of 19.3 to 29.7 °C for the control inoculant treated grass silage, a range of 18.9 to 30.0 °C across the five time points for the Ho LAB inoculant treated grass silage, and a range of 18.4 to 25.3 °C across the five time points for the He LAB inoculant treated grass silage.

4.3.4.8. Mycotoxin analysis of pre-ensiled grass and grass silage ensiled for 103 d after experiencing 0 or 8 days of aerobic exposure

There were only three mycotoxins identified in the pre-ensiled grass, that had been treated with either a control, Ho LAB or He LAB inoculant and in comparison, to silage either sealed after 24 h or sealed immediately for 103 days, where after 0 d of aerobic exposure, seven mycotoxins were identified, and after 8 d of aerobic exposure eight mycotoxins were identified (Table 4-9).

4.3.4.9. Deoxynivalenol

There was an effect of inoculant ($P = 0.011$) on DON concentration with the highest concentration of 71.3 µg/kg in Ho LAB treated silages, 65.3 µg/kg in control inoculated silages, and 55.8 µg/kg in He LAB treated silages (Table 4-9). There was also an effect of sealing time ($P = 0.004$) on DON concentration, where silage that experienced delayed sealing demonstrated a lower mean of 61.6 µg/kg in comparison to a mean of 77.6 µg/kg in silage that was sealed immediately. There was also an effect of time ($P < 0.001$) on DON concentration where pre-ensiled grass demonstrated a mean of 42.3 µg/kg, rising to a mean of 99.2 µg/kg after ensiling for 103 d without aerobic exposure (0 d) and lowering to a mean of 40.1 µg/kg after 8 d of aerobic exposure.

There was an interaction between inoculant and time ($P < 0.001$), sealing time and time ($P = 0.006$) and also an effect of inoculant, sealing time and time, ($P = 0.004$) with the highest mean concentration of DON at 0 d of aerobic exposure for the Ho LAB inoculant treated silage, that was sealed immediately (153 µg/kg), and the lowest mean concentration of DON at 8 d of aerobic exposure, for the control inoculant treated silage that was sealed immediately (18.6 µg/kg).

4.3.4.10. Enniatin B/B_1

There was an effect of time on enniatin B/B_1 concentration ($P = 0.048$; Table 4-9). Mean enniatin B/B_1 concentrations were highest in the pre-ensiled grass with a mean of 13.6 µg/kg, in comparison to a mean of 9.00 µg/kg in silage at 0 d of aerobic exposure, and 10.3 µg/kg in silage at 8 d of aerobic exposure.

There was an interaction between inoculant and sealing time ($P = 0.017$), and sealing time and time ($P = 0.006$) between the highest mean for enniatin B/B_1 of 19.1 µg/kg in pre-ensiled grass treated with He LAB in comparison to the lowest mean of 6.43 µg/kg in He LAB treated silage that was sealed immediately (Table 4-9).

4.3.4.11. Ergotamin(in)e

There was an effect of time on ergotamin(in)e concentration ($P < 0.001$), with silage after 103 d of ensiling, without aerobic exposure demonstrated the highest mean of 172 µg/kg, in comparison to a mean of 41.9 µg/kg after 8 d of aerobic exposure (Table 4-9). Pre-ensiled grass demonstrated no presence of ergotamin(in)e.

4.3.4.12. Fusaric acid

There was an effect of inoculant ($P < 0.001$) on concentrations of FUS, where mean concentrations were highest in Ho LAB treated pre-ensiled grass (109 µg/kg), followed by the mean concentration for control treated pre-ensiled grass (95.0 µg/kg) and the lowest in He LAB treated pre-ensiled grass (97.3 µg/kg; Table 4-9). There was no effect of sealing time ($P = 0.934$) as a lack of the presence of FUS in the silage meant that sealing time could not be evaluated. As a result of the absence of FUS in silage at 0 and 8 d of aerobic exposure, there was an effect of time ($P < 0.001$), and an interaction between inoculant and time ($P < 0.001$).

4.3.4.13. Fumonisin B_1

There was an effect of inoculant ($P < 0.001$) on fumonisin B_1, where the highest mean concentration of 24.2 µg/kg was demonstrated in silage treated with Ho LAB inoculant, followed by a mean of 11.3 µg/kg for silage treated with the control inoculant and the lowest mean concentration of 7.59 µg/kg for silage treated with He LAB inoculant (Table 4-9). There was no effect of sealing time ($P = 0.077$) on the concentration of fumonisin B_1, but there was an effect of time ($P < 0.001$), with the absence of fumonisin B_1 in the pre-ensiled grass, increasing to a mean of 5.04 µg/kg in silage after 0 d of aerobic exposure, and rising to a mean concentration of 23.7 µg/kg in silage after 8 d of aerobic exposure.

There was an interaction between inoculant and time ($P = 0.002$) and sealing and time ($P = 0.001$) with the highest mean concentration in Ho LAB treated silage after 8 d of aerobic exposure both with either a delayed sealing (44.3 µg/kg) or immediate sealing (41.1 µg/kg), in comparison to the lowest mean concentrations in He LAB inoculant treated silage after 0 d of aerobic exposure (4.07 µg/kg), and also after 8 d of aerobic exposure (6.40 µg/kg) both with immediate sealing (Table 4-9). There was a trend for an interaction between inoculant, sealing time and time ($P = 0.056$).

4.3.4.14. Fumonisin B_2

There was an effect of inoculant ($P = 0.015$) on fumonisin B_2, where the highest mean concentration of 9.48 µg/kg was demonstrated in silage treated with Ho LAB inoculant,

followed by a mean of 6.54 µg/kg for silage treated with the control inoculant and the lowest mean concentration of 4.10 µg/kg for silage treated with He LAB inoculant (Table 4-9). There was an effect of time (P = 0.007) also where fumonisin B_2, was not present in pre-ensiled grass, but was present in silage after 0 d of aerobic exposure with a mean of 5.81 µg/kg and also in silage after 8 d of aerobic exposure with a mean of 11.0 µg/kg.

There was an interaction between inoculant and time (P = 0.039), and inoculant, sealing time and time (P = 0.039) with a mean of 7.35 µg/kg and 9.00 µg/kg for control inoculant treated silage across 0 d and 8 d of aerobic exposure, respectively (Table 4-9). Mean concentrations of 5.90 µg/kg and 17.8 µg/kg were demonstrated in Ho LAB inoculant treated silage across 0 d and 8 d of aerobic exposure, respectively, and finally mean concentrations of 4.18 µg/kg and 6.08 µg/kg were demonstrated in He LAB inoculant treated silage across 0 d and 8 d of aerobic exposure, respectively.

4.3.4.15. Fumonisin B_3

There was an effect of sealing time (P = 0.029) on fumonisin B_3, with a mean concentration of 1.34 µg/kg for silage that experienced delayed sealing, and a concentration of 3.13 µg/kg for silage that was sealed immediately (Table 4-9). There was also an effect of time (P = 0.036) where pre-ensiled grass demonstrated a mean concentration of 0.00 µg/kg, silage that had experienced 0 d of aerobic exposure with a mean of 3.09 µg/kg and a lower mean of 1.38 µg/kg for silage that had experienced 8 d of aerobic exposure.

4.3.4.16. Moniliformin

Moniliformin was not present in pre-ensiled grass, or in silage after 103 days that had not experienced aerobic exposure (0 d) but was present in 5 silage treatments after 8 d of aerobic exposure, with a mean of 1.08 µg/kg (P = 0.002, Table 4-9).

4.3.4.17. Mycophenolic acid

Mycophenolic acid was not present in pre-ensiled grass, or in silage after 103 days that had not experienced aerobic exposure (0 d) but was present in only 3 silage treatments after 8 d of aerobic exposure, with a mean of 16.6 µg/kg (Table 4-9).

4.3.4.18. Penicillic acid

There was an effect of inoculant (P = 0.001) on mean PEN concentrations, with a mean of 1.01 µg/kg in silage treated with the control inoculant, a mean of 0.00 µg/kg for silage treated with the Ho LAB inoculant, and a mean of 11.0 µg/kg for silage treated with the He LAB

inoculant (Table 4-9). There was also an effect of sealing time (P = 0.008) with no presence of PEN in pre-ensiled grass, a mean concentration of 0.830 µg/kg for silage sealed that experienced delayed sealing, and the highest mean concentration of 9.17 µg/kg for silage that was sealed immediately.

There was an interaction between inoculant and sealing time (P = 0.007) with the highest mean concentration of 18.1 µg/kg in He LAB treated silage that was sealed immediately in comparison to 2.52 and 0.00 µg/kg for control and Ho LAB inoculated silage (Table 4-9). In silage that experienced delayed sealing, both control and Ho LAB inoculated silages had a mean of 0.00 µg/kg where, He LAB inoculant treated silages demonstrated a mean of 2.50 µg/kg.

4.3.4.19. Roquefortine C

Roquefortine C was not present in pre-ensiled grass, or in silage after 103 days that had not experienced aerobic exposure (0 d) but was present in silage treatments after 8 d of aerobic exposure, with a mean of 29.0 µg/kg (P = 0.050; Table 4-9).

Table 4-8. Dry matter loss, pH and temperatures of grass silage treated with either the control inoculant (no inoculant), Ho LAB inoculant, or He LAB inoculant with either immediate sealing (0 h) or delayed sealing (24 h) after 2, 4, 7, 16 or 26 d of aerobic exposure.

	Delayed sealing			Immediate sealing				Significance						
	Con	Ho LAB	He LAB	Con	Ho LAB	He LAB	s.e.d.	inoc	seal	time	inoc x time	seal x time	inoc x seal	inoc x seal x time
Dry matter loss (g)														
2 d	29.6^{a}	28.8^{a}	34.1^{a}	28.0^{a}	58.7^{a}	17.4^{a}	5.37							
4 d	207^{ab}	213^{ab}	77.5^{ab}	199^{ab}	214^{ab}	29.0^{a}	7.75							
8 d	346^{bc}	374^{bc}	219^{abc}	350^{bc}	344^{bc}	69.0^{cd}	7.99	< 0.001	0.031	< 0.001	0.011	0.692	0.027	0.930
16 d	645de	647de	474^{cd}	657de	650de	287^{abc}	13.0							
26 d	893^{ef}	932^{f}	752de	849^{ef}	921^{f}	641^{ef}	13.3							
pH														
2 d	3.60^{de}	3.54^{e}	3.77^{cde}	3.60^{de}	3.53^{e}	3.76^{de}	0.316							
4 d	7.50^{ab}	6.89^{ab}	5.20^{bcde}	6.54^{abcd}	6.75^{abc}	3.78^{cde}	1.410							
8 d	7.53^{ab}	7.91^{ab}	6.08^{abcde}	7.82^{ab}	7.87^{ab}	3.81^{cde}	1.100	< 0.001	0.043	< 0.001	< 0.001	0.655	0.059	0.842
16 d	8.11^{ab}	8.17^{ab}	7.36^{ab}	7.99^{ab}	8.04^{ab}	6.45^{abcde}	1.080							
26 d	8.22^{a}	8.04^{ab}	8.55^{a}	8.12^{ab}	8.48^{a}	7.75^{ab}	0.924							
Hours taken to reach 2 °C above ambient temperature														
2 d	37.1^{b}	NR	NR	45.5^{b}	43.4^{b}	NR	-							
4 d	59.1^{b}	62.9^{b}	72.2^{b}	65.1^{b}	55.3^{b}	NR	4.20							
8 d	53.6^{b}	51.3^{b}	52.6^{b}	54.4^{b}	47.9^{b}	187^{ab}	-	< 0.001	0.167	0.142	< 0.001	0.866	0.157	0.954
16 d	65.6^{b}	60.1^{b}	182^{ab}	67.3^{b}	57.0^{b}	247^{a}	8.89							
26 d	53.8^{b}	51.2^{b}	141^{ab}	56.3^{b}	46.8^{b}	239^{a}	7.04							
Maximum temperature reached (°C)														
2 d	20.0^{cd}	18.4^{d}	18.3^{d}	18.5^{d}	19.4^{d}	18.4^{d}	1.29							
4 d	28.9^{ab}	29.3^{ab}	23.9^{bcd}	28.6^{ab}	29.8^{ab}	18.8^{d}	2.00							
8 d	29.8^{ab}	30.8^{a}	24.0^{bcd}	29.6^{ab}	29.1^{ab}	19.1^{d}	1.72	< 0.001	0.005	< 0.001	< 0.001	0.679	0.056	0.612
16 d	30.1^{ab}	30.4^{ab}	26.1^{abc}	29.3^{ab}	29.6^{ab}	23.9^{bcd}	1.72							
26 d	29.8^{ab}	29.9^{ab}	26.1^{abc}	28.0^{ab}	29.9^{ab}	24.4^{abcd}	1.73							

NR = not reached, [1]Con = Control, [2]Homofermentative inoculant, [3]Heterofermentative inoculant, [4]inoc = Effect of inoculant, [5]seal = Effect of sealing time, [a-e]Superscripts denote values across rows and columns that are significantly different with Tukey's test.

Table 4-9. Mycotoxin content and significance for pre-ensiled grass or grass silage treated with either the control inoculant (no inoculant), Ho LAB inoculant, or He LAB inoculant with either immediate sealing (0 h) or delayed sealing (24 h) after 0 or 8 d of aerobic exposure.

Mycotoxins, µg/kg	Pre-ensiled grass			Silage, 0 d of aerobic exposure						Silage, 8 d of aerobic exposure						s.e.d.
				Delayed sealing			Immediate sealing			Delayed sealing			Immediate sealing			
	Con[1]	Ho LAB[2]	He LAB[3]	Con	Ho LAB	He LAB	Con	Ho LAB	He LAB	Con	Ho LAB	He LAB	Con	Ho LAB	He LAB	
Deoxynivalenol	44.4^{cde}	37.5^{de}	45.1^{cde}	95.4^{bc}	99.0^{bc}	56.6^{cde}	126^{ab}	153^{a}	64.9^{cd}	42.2^{de}	35.8^{de}	40.7^{de}	18.6^{e}	31.4^{de}	71.6^{cd}	31.18
Enniatin B/B_1	11^{ab}	10.7^{ab}	19.1^{a}	6.73^{ab}	10.2^{ab}	7.71^{ab}	11.1^{ab}	6.93^{ab}	11.3^{ab}	9.58^{ab}	14.0^{ab}	10.3^{ab}	11.2^{ab}	9.99^{ab}	6.43^{b}	5.67
Ergotamin(in)e	-	-	-	135	152	201	177	181	187	-	20.11	95.7	-	10.02	-	182.67
Fusaric acid	95.0^{c}	109^{a}	97.3^{b}	-	-	-	-	-	-	-	-	-	-	-	-	2.67
Fumonisin B_1	-	-	-	2.52^{c}	5.04^{bc}	-	12.2^{bc}	6.40^{bc}	4.07^{c}	30.6^{ab}	44.3^{a}	19.9^{abc}	-	41.1^{a}	6.40^{bc}	17.69
Fumonisin B_2	-	-	-	4.09^{b}	5.25^{b}	1.40^{b}	10.6^{ab}	6.55^{b}	6.96^{b}	11.5^{ab}	12.3^{ab}	8.06^{ab}	6.50^{b}	23.3^{a}	4.10^{b}	10.737
Fumonisin B_3	-	-	-	2.39	3.25	2.40	5.15	2.18	3.18	-	-	-	2.60	3.48	2.18	4.721
Moniliformin	-	-	-	-	-	-	-	-	-	0.418	1.08	0.850	1.61	1.43	-	1.585
Mycophenolic acid	-	-	-	-	-	-	-	-	-	3.23	41.8	-	4.84	-	-	38.18
Penicillic acid	-	-	-	-	-	5.00^{b}	5.03^{b}	-	13.8^{ab}	-	-	-	-	-	36.2^{a}	17.95
Roquefortine C	-	-	-	-	-	-	-	-	-	19.6	60.1	60.8	28.7	4.27	0.468	85.844
Risk equivalent quantity	8.60	8.80	9.20	48.0	53.7	68.6	67.1	66.1	71.9	21.0	86.2	78.0	27.2	9.48	33.3	194.95

Significance	inoc	seal	time	inoc x seal	inoc x time	seal x time	inoc x seal x time
Deoxynivalenol	0.011	0.004	< 0.001	0.232	< 0.001	0.006	0.004
Enniatin B/B_1	0.765	0.785	0.048	0.017	0.060	0.050	0.309
Ergotamin(in)e	0.527	0.789	< 0.001	0.556	0.999	0.380	0.949
Fusaric acid	< 0.001	0.934	< 0.001	0.983	< 0.001	0.860	0.981
Fumonisin B_1	< 0.001	0.077	< 0.001	0.423	0.002	0.001	0.056
Fumonisin B_2	0.015	0.164	0.007	0.380	0.039	0.296	0.039
Fumonisin B_3	0.828	0.029	0.036	0.719	0.742	0.230	0.468
Moniliformin	0.427	0.666	0.002	0.296	0.427	0.666	0.296
Mycophenolic acid	0.375	0.300	0.200	0.300	0.375	0.300	0.300
Penicillic acid	0.001	0.008	0.494	0.007	0.287	0.220	0.071
Roquefortine C	0.971	0.220	0.050	0.547	0.971	0.220	0.547
Risk equivalent quantity	0.560	0.316	0.226	0.836	0.843	0.133	0.635

"-" = mycotoxin was not detected, [1]Con = Control, [2]Homofermentative inoculant, [3]Heterofermentative inoculant, [4]inoc = Effect of inoculant, [5]seal = Effect of sealing time, [a-e]Superscripts denote values across rows and columns that are significantly different with Tukey's test.

4.3.5. Discussion

4.3.5.1. Treatment application and study design

The grass used in both experiments was made from a monoculture of *Lolium perenne* sward grown at Harper Adams University farm and harvested with a precision chop forage harvester. Field conditions and harvest management was therefore representative of on-farm conditions, which is important in improving the applicability of laboratory-scale silo studies to commercial farms (Cherney and Cherney, 2003). The application of an inoculant would usually occur during the harvest of the forage, as it is picked up in the field. However, in order to prevent cross contamination of treatments, the application of the additive was carried out by hand, using handheld pressure spray guns. The pressure of the spray gun may not however have replicated that used on-farm, although the rate of inoculant application was scaled to the volume of the forage to match that of the manufacturer's recommendations.

Many versions of mini-silo or laboratory silo have been used over time (McDonald et al., 1991). It was suggested by Cherney and Cherney (2003) that test tubes with a receptacle for the removal of silage effluent and valves for the escape of fermentation gases are most similar to the conditions experienced by silage on farm. However, often these experiments are designed to compare treatments where the aim is to alter the nutritional content of the silage rather than mycotoxin production. Moreover, the amount of forage that can be ensiled (approximately 250 g fresh weight) into a test tube is arguably too small to be representative of the compaction density observed within a standard grass silage clamp on a dairy farm in Great Britain (see Chapter 3). As continual measurements of the silage *in-situ* were not required in the current study, a destructive harvest design in closed glass jar mini-silos (Garber and Odland, 1927; Wang et al., 2014) was sufficient to assess the changes in the population of key microorganisms associated with mycotoxin production and silage fermentation.

In order to produce a volume of silage sufficient for the requirements of sampling for mycotoxin analysis, and wet chemistry analysis, whilst allowing leeway for expected DM loss during the aerobic stability experiment in part B (Borreani et al., 2018), twenty-four 30 L plastic barrel drums were also used to ensile the treatment forage. As the barrels were treated in the exact same way as the jar mini-silos, the wet chemistry analysis from the barrel silage was used to infer the probable proportions of fermentation end-products in the jar mini silos that were not measured, such as the lactic to acetic acid ratio. As the barrels differed in volume to the jars, the packing density used in the mini silo jars was matched as closely as possible when filling the barrels, with a mean of 600 kg fresh weight /m^3 in jars and a mean of 591 kg fresh weight

/m^3 in barrels, as silage density has been demonstrated to affect mycotoxin production by Gallo et al. (2018) and Snelling et al. (2023).

4.3.6. Silage fermentation profile

4.3.6.1. Silage pH, lactic acid and acetic acid production

The pH of the jar mini-silo silage in experiment A decreased from pH 5.41 to 3.75 within the first five days of ensiling, typical of that reported in grass silages (Haigh, 1987; Yin et al., 2023), and then maintained a stable acidic pH of pH 3.96 until opening at 98 d post-ensiling. According to the equation provided by Haigh (1987), a pH below 4.31 would be considered an effective fermentation for a forage with a DM of 244 g/kg pre-ensiling (Equation 12).

Equation 12. The theoretical maximum pH achieved by a forage during ensiling to provide an effective fermentation. Determined by Haigh (1987).

$$\text{Theoretical pH maximum} = 0.00359 \times \text{Dry matter (g/kg)} + 3.44$$

There was no effect of silage inoculant on the rate at which the pH declined in the silage experiment A which is in contrast to reports that indicate obligate heterofermentative directed pathways may lead to a slower pH decline in silage, as demonstrated in Wang et al. (2014). This is due to the production of only one mole of lactic acid and one mole of acetic acid per mole of hexose sugar, in comparison to the homofermentative pathway that produces two moles of lactic acid per hexose sugar (McDonald et al., 1991). In support of this, the barrel silage in experiment part B, did demonstrate an effect of inoculant as was expected, with a higher pH in the He LAB treated silage, yet this was still at a mean value of 3.68, and therefore still below the pH value of 4.31, indicating a successful fermentation. The enclosed system of the jar and barrel mini-silos also prevented effluent from leaving the immediate silage environment, which may have increased the efficiency of pH reduction compared to a silage pit on farm, where the effluent is able to drain out and away from the silage (Jones and Jones, 1995).

The He LAB inoculant used in the current study contained only one species of obligate homofermentative bacteria: *Pedioccocus pentosaceus,* however this was included at a lower CFU than that of the two obligate heterofermentative bacteria also within the He LAB inoculant. This was likely to have been insufficient to have had an effect on the overall production of lactic acid in the jar mini-silos in part A (Holland et al., 2021) and was demonstrated as such in experiment part B by a lower lactic:acetic acid ratio for barrel silage treated with He LAB inoculant (2.12) in comparison to Ho LAB and Control silage (7.27 and 5.56, respectively).

Interestingly, sealing time had no effect on the rate of pH reduction of the grass silage in part A, despite evidence that a delayed sealing time on-farm can disadvantage the silage fermentation (Pahlow et al., 2015; Weiss et al., 2016; Wilkinson and Davies, 2013). Likewise, there was no difference in pH at 103 d post ensiling in barrel silage as a result of sealing time. It may be argued that the silage density was high enough in the mini-silos (591 to 600 kg fresh weight /m^3) that even with the lid of the jar or barrel unfastened, the silage may have maintained a highly anaerobic environment where only the top of the jar or barrel was impacted. In comparison, the median densities achieved on farms visited in Chapter 3, were between 499 and 662 kg fresh weight /m^3, but in some cases, were as low as 157 and 177 kg fresh weight /m^3 in the left and right shoulders, respectively. As forage was packed by hand, variation between individuals may have affected the homogeneity of the silage density. Certainly, this was visibly the case for some of the jars in part A that had experienced delayed sealing due to a clear distinction of areas of top spoilage (Figure 4-4). However, as air circulation within mini silos was not measured it was not possible to conclusively determine the depth to which oxygen infiltrated the forage mass.

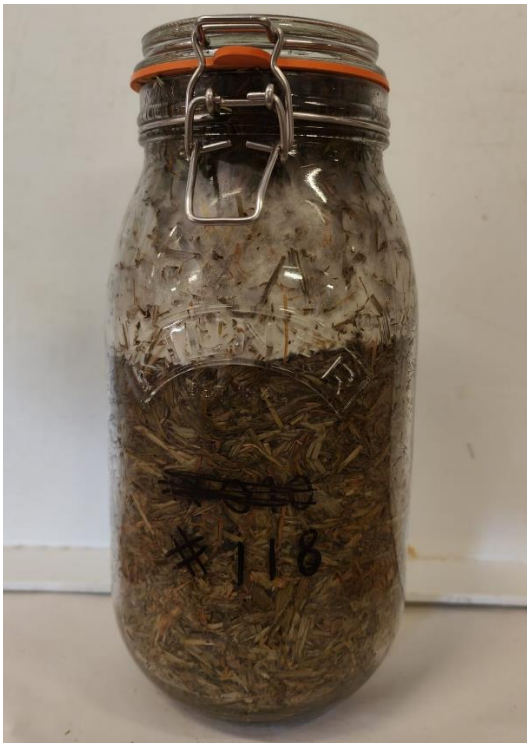


Figure 4-4. One of the mini-silo jars that had experienced delayed sealing, demonstrating a clear difference between the visible spoilage at the top of the jar, in comparison to the lower part of the jar.

4.3.6.2. DM and DM loss

The DM of the silage in part A was unaffected by inoculant treatment and sealing time, however the mean DM loss was. As expected, the delay in sealing the silos resulted in a greater loss of DM from ensiling to 98 d post ensiling, reiterating that clamp management can impact on silage fermentation (Muck, 1988; Weiss et al., 2016). The He LAB inoculant treated silage experienced the greatest DM loss (32.3 g), supporting that the obligate heterofermentative pathway leads to a greater loss of nutrients through the fermentation pathway and production of CO_2 gas (McDonald et al., 1991; Wróbel et al., 2023). Furthermore, the DM content of the barrel silage in part B after 103 d post ensiling, was shown to be lowest in the He LAB treated silage.

4.3.7. Silage microbial profile and population counts

4.3.7.1. Enterobacteriaceae

In part A, counts of *Enterobacteriaceae* were not affected by the inoculant that was added, suggesting that both the lactic acid produced in the silage treated with Ho LAB, and the lactic and acetic acid produced by He LAB were both as effective at reducing the pH and inhibiting *Enterobacteriaceae* growth in grass silage. This is supported by the lack of a difference between the two inoculants on the pH. There was a trend (P = 0.070) for an effect of sealing time on the counts of *Enterobacteriaceae*, with a higher mean population in silages that had delayed sealing (4.70 $\log_{10}$ CFU/g FW, or 5.01 x 10^4 CFU/g FW) compared to those sealed immediately (3.88 $\log_{10}$ CFU/g FW or 7.59 x 10^3 CFU/g FW) This again supports that delayed sealing of a silage clamp can increase spoilage organism proliferation during fermentation (Henderson and McDonald, 1975; Wróbel et al., 2023).

4.3.7.2. Lactic acid bacteria (LAB)

There was no effect of inoculant on LAB colony abundance in jar silage in part A, suggesting that the epiphytic population of LAB on the fresh grass was sufficient (approximately 1.48 x 10^6 CFU/g FW) to ensure an effective fermentation without the addition of either a Ho LAB or He LAB inoculant. This finding is in contrast to that reported by Carvalho et al. (2021) and Wróbel et al. (2023), and a study by Cai et al. (1999b) suggested that epiphytic LAB are often found at levels no greater than approximately 1.00 x 10^5 CFU/g fresh substrate.

By 2 d post ensiling, the LAB abundance had risen to approximately 1.41 x 10^9 CFU/g FW in the Control, similar to that of the Ho LAB inoculant treated silage ~ 1.32 x 10^9 CFU/g FW, and the He LAB inoculant treated silage ~ 9.33 x 10^9 CFU/g FW. A much higher colony count may

have been expected at 2 d, with the addition of the LAB inoculants due to the application increasing initial LAB load. The total bacteria applied to the forage in the He LAB inoculant was: *Lentilactobacillus hilgardii:* 2.0 x 10^5 CFU/g FW, *Lentilactobacillus buchneri* 2.0 x 10^5 CFU/g FW and *Pediococcus pentosaceus* 1.0 x10^5 CFU/g FW, and in the Ho LAB inoculant: *Lactiplantibacillus plantarum*: 5.0 x 10^5 CFU/g FW, and *Pediococcus pentosaceus:* 5.0 x 10^5 CFU/g FW. However, intermicrobial competition, and diurnal variation (Dong et al., 2023) within epiphytic forage populations, ambient temperature at harvest, the water soluble carbohydrate content of the forage and general loss of viable bacteria in the freeze drying and storage process of the inoculants (Miyamoto-Shinohara et al., 2006), would all impact on the resultant LAB abundance at 2 d post ensiling. Interestingly, Wróbel et al. (2023) suggested that second cut grasses in Poland, would be most likely to harbour the highest abundance of epiphytic LAB due to the grass entering the middle of the growing season. A greater differentiation between epiphytic populations and the Ho LAB and He LAB inoculated silage may therefore have been observed had the forage used been first or third cut.

In contrast, levels of LAB at 103 d post ensiling in part B, were affected by inoculant, with the highest mean LAB count of 7.97 x 10^7 CFU/g FW in He LAB treated silage in comparison to Control or Ho LAB treated silage (1.15 x 10^7 CFU/g FW and 1.46 x 10^6 CFU/g FW, respectively). This may have been as a result of the greater LAB load provided initially by the He LAB inoculant, however the intermediate value for the Control seems to support that this is due to the secondary fermentation activity of obligate heterofermentative LAB of lactic acid (S. J.W.H. Oude Elferink et al., 2001), and is in agreement with the concentration of propan-1-ol and propane-1,2-diol found at highest concentrations in He LAB treated silage. It is therefore possible that populations of LAB within the He LAB inoculant were able to sustain a higher abundance for longer through the utilisation of lactic acid present. Furthermore, water soluble carbohydrate concentration of the barrels in part B were reduced for He LAB inoculant silages in comparison to the control and Ho LAB treated.

4.3.7.3. Filamentous fungi

The abundance of filamentous fungi in jar silage of part A and barrel silage of part B, was not impacted by the addition of an inoculant, which agreed with findings of Wang et al. (2014). As acetic acid has been demonstrated to inhibit fungal growth (Danner et al., 2003), a lower fungal abundance may have been expected with the silage treated with He LAB inoculant in comparison with the Ho LAB and Control, but this was not so. It is important to note that culturing fungi on MEA with the addition of chloramphenicol is promotive to the growth of fungi (Black, 2020; Skaar and Stenwig, 1996), and may have led to an overestimate of the fungal population.

A delayed sealing time led to an increase in the abundance of filamentous fungi in part A, in comparison to silage that was sealed immediately, despite the establishment of an acidic pH across both immediately and delayed sealing treated silage that was able to inhibit bacterial growth. This suggests that fungal growth is not inhibited to the same extent that spoilage bacterial organisms are due to pH reduction alone, possibly explained by the ability of fungal organisms to modulate their immediate environmental pH (Vylkova, 2017) and supported by Wheeler et al. (1991) whom demonstrated the ability of isolates of *Fusarium, Aspergillus* and *Penicillium,* to grow under pH values ranging from 2 to 11. The duration of oxygen exposure in silages therefore plays a larger role in influencing fungal proliferation.

Nevertheless, even in immediately sealed silage there was still an increase in fungal populations from a mean of ~114 CFU/g FW at 2 d post ensiling to a mean of ~ 3.02 x 10^3 CFU/g FW at 98 d post ensiling, suggesting that despite a more rapid reduction in available oxygen at the beginning of the fermentation, fungal populations were able to survive and regrow from 5 d post ensiling onwards. This is in agreement with McCullough et al. (1986) whom states that some filamentous fungi are able to survive under low oxygen concentrations. This study supports the idea that mycotoxin formation during this phase of ensiling is therefore still possible, as populations of fungi are present, and mycotoxins may even be synthesised as a response to increasing lactic and acetic acid concentrations across 2 d to 5 d post ensiling (Alcano et al., 2016; Vylkova, 2017) as was the relationship with the REQ described in Chapter 3. As this coincides with the increase in abundance of LAB, it could be that mycotoxins are produced in response to antimycotic compounds produced by LAB (Gourama and Bullerman, 1995; Khalil et al., 2013).

4.3.7.4. Yeasts

Yeast populations in experiment A mini-silos were affected by the inoculant used, and there was an interaction with time, where silage treated with the He LAB inoculant had a lower yeast population at 98 d post ensiling, in agreement with the negative impact of acetic acid on yeast growth (Danner et al., 2003; Guaragnella and Bettiga, 2021). This is echoed by the varying yeast counts across inoculant treatments where varying proportions of homolactic and heterolactic LAB populations would have been present (S. J.W.H. Oude Elferink et al., 2001). Moreover, the barrel silage in experiment B, demonstrated an effect of inoculant on yeast count reflective of that of the mean ratio of lactic:acetic acid, which was 7.27 in Ho LAB treated silage, 5.56 in Control and 2.21 in He LAB treated silages; in agreement with findings from other ensiling studies such as Wang et al. (2014).

In part A, there was no effect of sealing on yeast counts in silage, however the barrel silage in part B, demonstrated an interaction between inoculant and sealing time, where lower yeast abundance was observed in silage that had been sealed immediately in comparison to a delayed sealing, both with He LAB inoculant. Numerically however this resulted in a difference of only 581 CFU/g FW; arguably counts of yeast in the He LAB silage that were sealed immediately were too low to discern a meaningful biological difference and are likely due to overestimations in plate counting.

It is interesting to observe that though both fungi, yeasts and filamentous fungi in the silage seem to have been affected differently by inoculant and sealing time. This is likely due to the physiological, morphological and biochemical differences between the unicellular yeasts and multicellular filamentous fungi (Powers-Fletcher et al., 2016). There is little information on a direct comparison of sensitivities to acetic acid that the two groups express, in part due to the vast diversity within and between these groups (Kawahata et al., 2006). Though acetic acid is effective in maintaining aerobic stability (Danner et al., 2003) as demonstrated to lead to apoptosis in yeast (Ludovico et al., 2001), a study by Fernández-Niño et al. (2015) reported that some colonies of *Saccharomyces cerevisiae* display acetic acid tolerance dependent on their cytosolic pH level, and recently another yeast species proving problematic to the wine and baking industries is *Zygosaccharomyces bailii*, which is able to tolerate high levels of acidity and lead to spoilage of these products (Fernández-Niño et al., 2015; Stratford et al., 2013).

Mixed tolerance ability of acetic acid is also demonstrated in filamentous fungi. For example, Cabo et al. (2002) demonstrated that acetic acid produced by LAB resulted in a reduction in growth of *Penicillium discolor,* and Alcano et al. (2016) showed that *Aspergillus* (section *Nigri*) spp. became more sensitive to acetic acid as pH decreased from pH 6.0 to 4.5. Interestingly the authors also demonstrated an ability of sorbic acid “under-dosing” (where growth is mildly inhibited) to increase production of the mycotoxin Ochratoxin A, in *Aspergillus niger*. In contrast, and most applicable to this study, Boysen et al. (2000) stated that cultures of *Penicillium expansum* isolated from grass silages were able to grow on 0.3 % (v/v) acetic acid (approximate pH of 3.0), but were sensitive to CO_2 levels higher than 15 %, indicating tolerance to acidic conditions, but sensitivity to oxygen exposure. This is in support of the findings of this study. Further research would be required to identify the exact filamentous fungal and yeast species present in the grass silage to determine specific sensitivities.

4.3.8. Aerobic stability of silage

4.3.8.1. DM loss, pH and temperature changes

The aerobic stability assessment in part B showed that the least DM lost was from the He LAB treated silage at 2, 4, 8 and 16 days of aerobic exposure with either sealing time, as was expected (Danner et al., 2003; Nadeau et al., 2018) and supported by the maintenance of an acidic pH of around 3.78 from 2 to 8 d post exposure. However, by 26 d post exposure there were no differences between DM lost between the Ho LAB, He LAB and control treated silages. This was supported by the changes observed in pH for the He LAB treated silage. At 16 post exposure, the pH of immediately sealed He LAB treated silage reduced in acidity to match that of the neutral pH observed in the Ho LAB and Control silages (mean pH of 7.68). In delayed sealing, this reduction in acidity in the He LAB silages was observed by only 4 d of air exposure. If a silage therefore experiences delayed sealing on-farm, and this particular He LAB inoculant was applied with the intention of improving aerobic stability of the silage, a 4 d window may not afford a farmer long enough to match the duration of the feed out rate, to prove beneficial. As soon as pH reduces in acidity, spoilage organisms are able to proliferate rapidly and in Chapter 3, the reported feed out times ranged from 2 to 8 days, indicating that some farmers took longer than 4 days to feed out across the clamp face completely.

It is important to note, however, that the rate of aerobic spoilage of silage demonstrated in this trial does not take into account compaction and clamp management, which would aid in preventing oxygen ingress into the silage (Wilkinson and Davies, 2013), as silage compaction density was lost when the silage was placed into the paint tins. Thought the rate may not be comparable to on-farm conditions, it is still comparable between that of the other treated silages within the experiment.

Temperature can be used as a proxy for microbial activity, and the time taken to reach 2 °C above the ambient temperature is used as a measure of aerobic stability by other studies such as that of Ranjit and Kung (2000) and Ferrero et al. (2021). Again, as expected the time taken for temperatures to increase 2 °C above the ambient temperature was longest for He LAB treated silage with a mean duration of 190 h after 26 d of exposure. There were no differences seen between the duration of time taken to reach 2 °C above ambient or DM loss between the Control and Ho LAB treated silage, despite reports by Danner et al. (2003) and Weinberg et al. (1993) that Ho LAB treated silage displays accelerated aerobic spoilage in comparison to control; however Weinberg et al. (1993) argued this is dependent on the concentration of water soluble carbohydrates, acetic acid and VFAs present in the silage, and the study was based

on wheat and sorghum silages. Furthermore, a study by Cai et al. (1999a) on Italian ryegrass silage displayed no difference in DM loss between a Ho LAB inoculant and the control.

4.3.8.2. Yeast and filamentous fungi abundance and mycotoxin content

During the assessment of aerobic stability in part B, for both fungal and yeast counts, there was an increase in abundance across days of exposure as was expected (Weiss et al., 2016). At four days of exposure, fungal abundance was lowest in He LAB treated silages that were sealed immediately (3.98×10^3 CFU/g FW), which drove a difference in the lower overall mean in comparison to the other treated silages, however by 16 d of exposure, the CFU of fungi were at similar levels to all other treated silages, with an overall mean of 1.00×10^8 CFU/g FW.

4.3.8.3. Changes in mycotoxin profile across time

The numerical concentrations provided from the mycotoxin tests are a mean of three analytical replicates and can be influenced by sampling method, despite steps taken to homogenise samples before analysis. Due to the values being within the same magnitude as one another and at generally low levels of contamination, the variability across the treatments is not different enough to conclude a biological effect of inoculant treatment, sealing time or aerobic exposure on the concentration of mycotoxins, despite statistical difference seen. The presence and absence of mycotoxins, however, can provide an overall depiction of the shifts in mycotoxin presence and absence in the grass, to silage and to aerobic spoilage stages of the experiment. Fresh grass contained only three mycotoxins pre-ensiling, DON, enniatin B/B_1 and FUS which are known to be produced by *Fusarium* fungi and are therefore generally considered to be field-formed mycotoxins (Johns et al., 2022; Perincherry et al., 2019). Consistent with the findings in Chapter 3, DON was present in the silage but was present at levels below that of the EU recommendations (European Union (EU), 2006).

Fumonisins and moniliformin are also synthesised by *Fusarium* species but were not present in the pre-ensiled grass. Concentrations of FUM varied greatly between silage inoculant and presence or absence at post ensiling or after 8 d of aerobic exposure. As with most other mycotoxins, FUM are considered thermally stable and are largely unaffected by pH (Bryła et al., 2017), therefore any subsequent absence from a silage previously found to contain the mycotoxin suggests modification by microbial processes. There is known effect of fumonisin B_1 as an antifungal agent that inhibits growth of *F. expansum*, *F. graminarium* and *Alternaria alternata* (Keyser et al., 1999) suggesting that the shifts in specific mycotoxin synthesis across

time are as a result of complex intermicrobial competition for resources (Rohlfs et al., 2007; Venkatesh and Keller, 2019).

Interestingly, ergotamin(in)e is also considered a field formed mycotoxin of the *Claviceps* fungi, but ergotamin(in)e was not present in any of the grass pre-ensiling, and appeared after fermentation. Roberts et al. (2014) reported an inconsistent presence and absence of different ergot alkaloids when studied over a time course in fescue before and after ensiling. This presence and absence across time was observed in this study as ergotamin(in)e appeared during ensiling and then decreased in concentration during aerobic exposure, and in some instances disappeared altogether.

Another mycotoxin that appeared during the ensiling process was PEN. Produced by *Penicillium* species, this was in line with the prevalence of PEN found in over 70% of silage samples in Chapter 3, and in agreement with the various *Penicillium* fungi isolated from grass silage previously (O'Brien, 2010; O'Brien et al., 2006). As PEN has been found to disrupt quorum-sensing between bacteria, as a competitive strategy (Rasmussen et al., 2005), it may be hypothesised that the increased microbial activity of LAB during the fermentation process could trigger the production of PEN by *Penicillium* fungi present on the forage.

After 8 days of aerobic exposure, roquefortine C, and MPA appeared, consistent with O'Brien et al. (2008) that suggest these *Penicillium* associated mycotoxins likely appear during aerobic spoilage of grass silage. Interestingly it seems that PEN was not found in most samples of silage after 8 d of aerobic exposure, indicating a potential shift in either *Penicillium* species abundance, or a shift in the specific mycotoxins produced by a dominant *Penicillium* species in the silage at this time point. There is little information concerning the exact mycotoxin shifts and their cause and further work could aim to elucidate this difference. Either way, this study demonstrates that the mycotoxin content of silage is dynamic and is likely as a result of the complex interactions between microorganisms in the silage as they compete with one another for resources (Venkatesh and Keller, 2019).

4.3.8.4. Fermentation profile and mycotoxin risk

The differing fermentation profile of the silages had no effect on the overall mycotoxin risk of the grass silage, and this may be explained by a lack of difference between initial DM content of the forage ensiled and even the calculation of the REQ. For example, DM content was able to explain over 50 % of the variation in mycotoxin risk in Chapter 3, however all forage ensiled in this study was already of a lower DM (244 g/kg in fresh grass) and so differences in resultant silage mycotoxins as a result of inoculant treatments may have been masked. However,

despite a lower DM silage and high lactic and acetic acid content of the He LAB silage, the majority of forage was identified as low risk, according to the REQ. An REQ > 100 µg/kg is considered moderate risk and > 150 µg/kg is considered high risk for an impact on the performance of mature dairy cows by Alltech©, however only two of the individual silages were over 150 µg/kg threshold, and this was likely driven by the DON or FUM content in these samples, as the effects of these metabolites on ruminants have been better studied (Seeling and Dänicke, 2005). Overall, the mycotoxin content of the silages was low, despite the correlation found between lower DM content and higher risk REQ (Chapter 3), suggesting that other factors such as field management, clamp management and ensiling methods are also key factors in determining the mycotoxin content of a silage, not just the fermentation profile. A significant effect of time was found for nine mycotoxins out of the eleven, highlighting the necessity for multiple mycotoxin sampling points across time from field to feed out, in order to better gauge the current risk posed to cattle. Furthermore, the study also reiterates that there is no correlation between mycotoxin content of a silage and the abundance of visible fungi, as was also demonstrated by (Manni et al., 2022).

4.3.9. Conclusions

There was a high enough lactic acid bacteria abundance by 2 d post ensiling, to lower pH sufficiently enough to reduce the population of *Enterobacteriaceae*. The addition of He LAB increased DM lost by 98 d post ensiling, likely due to the activity of obligate heterofermentative bacteria. Directing the fermentation towards a more lactic acid or combination of lactic and acetic acid pathway did not affect the abundance of filamentous fungi, however a delayed sealing time resulted in a greater fungal population at 98 d post ensiling. Despite the reduction in pH and establishment of lower oxygen levels more rapidly in silage that was sealed immediately, viable fungi were still present from 2 d to 98 d post ensiling. Yeast counts were unaffected by sealing time but there was a reduced population at 98 d post ensiling in He LAB inoculant treated silage. Yeasts and filamentous fungi responded to inoculant treatment and sealing time differently. Mycotoxin production was not correlated with fungal abundance, and concentrations of specific mycotoxins varied across time. Despite high fungal abundance during the period of aerobic exposure, mean mycotoxin REQ remained low risk for all silages.

Chapter 5

The impact of grass silage mycotoxins at three levels of inclusion, alone or in combination with one another on the rumen microbial metabolism *in-vitro.*

5. The impact of grass silage mycotoxins at three levels of inclusion, alone or in combination with one another on the rumen microbial metabolism *in-vitro.*

5.1. Introduction

The most common mycotoxins reported in grass silages from the literature (Driehuis et al., 2008; O'Brien, 2010), on GB farms (Chapter 3) and in mini silo studies (Chapter 4) are mycophenolic acid (MPA), penicillic acid (PEN), and fusaric acid (FUS), with deoxynivalenol (DON) and zearalenone (ZEA) occasionally being detected. The effects of DON and ZEA on cattle health and dairy cow performance have been more comprehensively studied due to their acute toxicity and greater prevalence in grain and maize silage (Elweza et al., 2022; Gnezdilova et al., 2023; McKay et al., 2019). Additionally, the ability of the rumen microbial community to degrade DON and ZEA has also received greater research attention.

Despite the lack of evidence in the literature for acute toxicity associated with the mycotoxins more commonly found in grass silage, they could still be detrimental to dairy cow performance, (Fink-Gremmels, 2008). Exposure to these toxins may lead to chronic issues, where often it cannot be conclusively determined that mycotoxins are the cause (Gallo et al., 2022). As FUS, MPA and PEN possess antibacterial properties (Bentley, 2000; Geiger and Conn, 1945; Zhang et al., 2021), they may disrupt the rumen microbial community (Guerre, 2020) consequently affecting ruminal pH, VFA production and the general health of the cow. Some mycotoxins have also been found to work synergistically alongside others (Speijers and Speijers, 2004) either lessening or heightening the risk to cow performance and health.

Studies that have investigated mycotoxin – rumen microbial interactions, have most often focussed on the ability of certain microbial taxa to degrade mycotoxins into less toxic forms (Adegoke et al., 2023; Guerre, 2020; Min et al., 2021). Guerre, (2020) suggested that this was due to the greater complexity of characterising the effect of mycotoxins on the microbial community and is supported by the lack of published studies in this area (Gallo et al., 2022). Two recent studies by Hartinger et al., (2023, 2022) that did investigate the effect of mycotoxin inclusion on the rumen microbial composition in dairy cows, involved a ZEA - bolus and ZEA and FUM added to a moderate-grain based diet, respectively. There are therefore no studies to date that have investigated the effect of FUS, MPA or PEN that are most commonly found in grass silage, on rumen microbial metabolism.

5.2. Hypotheses and objectives

The hypothesis was that mycotoxins commonly found in grass silage would affect rumen metabolism *in-vitro* when fed alone or in combination with each other.

The objectives of the studies were to determine the effect of four grass silage mycotoxins (FUS, PEN, MPA and DON) at

i) three levels of inclusion (medium, high and extreme),
ii) or in combination with one another (all at a medium level of inclusion),

on rumen metabolism and microbial activity *in-vitro*

The objectives were tested via a combination of *in-vitro* studies, including a consecutive batch culture (CBC) method with fifteen generations, and three batch culture *in-vitro* fermentation model (IFM) incubations for 48 h. A CBC and IFM pilot study were undertaken to optimise the fermentation conditions.

This chapter contains 3 sections:

i) Pilot experiment (CBC and IFM combined method)
ii) Method development and optimisation
iii) Two *in-vitro* fermentation model (IFM) experiments

5.3. Pilot experiment: CBC and IFM evaluation of the effect of three levels of inclusion of four grass silage mycotoxins on rumen fermentation

The pilot CBC experiment used consecutive batch cultures (each batch labelled as a "generation") of 125 ml glass serum bottles (fermenters) filled with 100 ml of rumen fluid and buffer (in a 1:9 ratio) treated with four mycotoxins (FUS, PEN, MPA and DON), at three levels of inclusion (medium, high and extreme), with 1 g of milled and dried grass silage. Every five CBC generations an IFM evaluation was undertaken (48 h incubation at 39°C) using culture from the CBC, yielding a total of three IFM runs (15 generations in total).

By CBC generation 5, it became apparent that the microbial activity of the inoculum was too poor to sustain any further viable generations, and so the study ceased until further method development and optimisation. The pilot study findings have been outlined below.

5.3.1. Pilot experiment: Materials and methods

5.3.1.1. Treatments

Four common grass silage mycotoxins were used: Mycophenolic acid (MPA), penicillic acid (PEN), fusaric acid (FUS), and deoxynivalenol (DON; Table 5-1). Levels of inclusion were classed as “medium”, “high”, and “extreme”. The “medium” inclusion level was informed by median concentrations of each mycotoxin determined in the grass silage samples from the farms surveyed in Chapter 3 (Appendix Table 4) .“High” levels of inclusion were informed by the mean concentrations for each mycotoxin found in grass silage samples in Chapter 3, and “extreme” levels of inclusion, as exceeding naturally occurring concentrations. All concentrations were calculated with the expectation that the three levels of inclusion would affect rumen microbial metabolism.

Table 5-1. Treatment outline for the pilot consecutive batch culture study *in-vitro* to determine the effect of four grass silage mycotoxins at three levels of inclusion on rumen fermentation

Treatment code	Treatment details	Level of inclusion/details	Mycotoxin µg/g DM	Ethanol[1] ml/g DM
Control	100% Ethanol	Negative Control	-	2.00
MPA	Mycophenolic Acid	Med	1.00	1.00
	Mycophenolic Acid	High	5.00	1.50
	Mycophenolic Acid	Extreme	25.00	2.00
PEN	Penicillic Acid	Med	1.00	1.00
	Penicillic Acid	High	4.00	1.50
	Penicillic Acid	Extreme	16.00	2.00
FUS	Fusaric Acid	Med	1.00	1.00
	Fusaric Acid	High	4.50	1.50
	Fusaric Acid	Extreme	20.25	2.00
DON	Deoxynivalenol	Med	0.500	1.00
	Deoxynivalenol	High	2.25	1.50
	Deoxynivalenol	Extreme	10.00	2.00

[1]The volume of ethanol that was added to solubilise the mycotoxin.

5.3.1.2. Setup of the consecutive batch culture

The method for the consecutive batch culture was adapted from Gascoyne and Theodorou, (1988) and Davies, (1991). The buffer used in the consecutive batch culture (Buffer medium C) was adapted from Orpin, (1976) as described by Davies, (1991) and is detailed in Appendix Table 3. Rumen fluid was collected as described in Section 5.3.1.4, and kept on ice, before centrifugation at 25000 xg for 20 mins at 4 °C according to Bryant and Robinson, (1961) to form the clarified rumen fluid for buffer medium C.

Clear 125 ml Wheaton serum bottles (DWK Life Sciences, Germany) were filled with 1 g of dried and milled grass silage, obtained during the farm survey carried out in Chapter 3; Table 5-2. When making up buffer medium C (Appendix Table 3) constituents were added to a 5 L duran bottle in a water bath at 80 ºC, with gassing with a sintered thimble attached to a CO_2 canister (BOC, Woking, United Kingdom; Figure 5-1). When adding the salt solutions I, II (Appendix Table 3) and clarified rumen fluid to form the buffer, they were first microwaved on low power, until bubbles appeared on the surface of the liquid, before addition. Distilled water was boiled in a kettle before, being left to cool to approximately 70 ºC before addition. Once complete, the buffer spent a further 1.5 hours gassing under CO_2 gassing in a water bath at 40 ºC.

A peristaltic pump (Jencons Perimatic, Bedfordshire, United Kingdom) was used to dispense 90 ml of buffer medium C into each serum bottle, with the bottles flushed with CO_2 via four 16 G gassing needles attached to “Y” adaptors for approximately 10 seconds each (Figure 5-2). After dispensing, bottles were sealed rapidly with butyl rubber bungs (Chemglass Lifescience LLC, United States) and crimped into place with aluminium crimp caps (Chromacol, Thermo Fisher Scientific, United States). Sealed bottles were autoclaved at 121 ºC for 15 min and then incubated at 39 ºC before the addition of the mycotoxin treatment and subsequent fresh rumen fluid inoculum.

5.3.1.3. Mycotoxin application

Working concentrations of all mycotoxins were formed by eluting the crystalline mycotoxin into 100 % ethanol and vortexing thoroughly to solubilise. Working concentrations were stored at -20 ºC between use. PEN (Cat: APOSBIP1005) and DON (Cat: 89153-032) were obtained from VWR® (Avantor, Pennsylvania, United States) whilst MPA (Cat: 459380250), and FUS (Cat: 198960010), were obtained from Acros® Organics (Thermo Fisher Scientific, Massachusetts, United States).

Under aseptic conditions, using a 2 ml graduated luer lock syringe (BD, New Jersey, United States), and 20 G needle tip (BD, New Jersey, United States), sterile serum bottles were injected through the butyl bung with the respective concentration of one of the four treatment mycotoxins at three levels of inclusion (Table 5-1). For the negative control treatment, 2 ml of 100% ethanol was injected into the serum bottles. Bottle bungs were flamed quickly with a Bunsen burner post-injection to ensure resealing of the butyl rubber after puncture. Injection of mycotoxins occurred no earlier than 4 hours prior to inoculation of the serum bottles with rumen fluid. Bottles were kept in an incubator at 39 ºC after mycotoxin injection and before rumen fluid inoculation. Three replicates per treatment were assigned, with a total of 39 bottles per inoculation batch (referred to as a "generation").

5.3.1.4. Inoculation with rumen fluid

All procedures concerning the use of animals for this research, were conducted in accordance with the Animals (Scientific Procedures) Act of 1986 (amended 2012) of the United Kingdom (available at: www.legislation.gov.uk/ukpga/1986). All procedures were also approved by the Harper Adams University local ethics committee.

Three Holstein – Friesian dairy cows that had been fitted with a 20 cm diameter rumen cannula were restrained in mattress bedded stalls at Harper Adams University Dairy Cow Metabolism Unit (Figure 5-3). The cows were milked in the stalls daily and so were familiar with surroundings. Fresh water was available at all times during restraint, and the cows were restrained for a duration of no longer than one hour per rumen fluid sampling session. The cannula plug was removed by gloved hand from the cannula by gently pushing in a forward direction. Using a 250 ml Duran bottle (DWK Life Sciences, Germany) rumen fluid was collected by entry of the bottle into the rumen through the cannula and submerging within the liquid phase of the ventral region (below the dorsal mat) of the rumen (Figure 5-3). Rumen fluid was collected at an approximate depth of 60 cm from the top of the rumen and immediately strained through four layers of muslin, to remove solid digesta, into a 1 litre thermal flask (Thermos®, West Yorkshire, United Kingdom). The flasks were filled with distilled water at 39°C to create an anoxic environment and emptied immediately prior to filling with sample rumen fluid. The volume of the flask was exceeded with rumen fluid to ensure no head space gas remained to aid in maintaining anaerobic conditions, before screwing the lid of the flask. Approximately 3 litres total of strained rumen fluid were collected. Rumen fluid pH for each cow was recorded immediately post collection using excess strained sample poured into a clean 250 ml plastic cup and determined using a calibrated benchtop pH meter (Jenway 3510, Antylia Scientific, Illinois, United States). No more than 2 litres of rumen fluid were removed from an individual cow during any sampling.

For all *in-vitro* studies, rumen fluid was collected 3 hours after feeding. Rumen fluid from the three cows was mixed proportionally (1:1:1) in the laboratory into a clean 5 L duran bottle, held in a water bath at 39 ºC under CO_2 to provide a single combined inoculum.

Using a 10 ml graduated luer lock syringe and 20 G needle tip, 10 ml of rumen fluid was injected into each bottle under aseptic conditions. Bottle bungs were flamed before and after inoculation and the bottles were returned to a 39°C water bath immediately. Once all bottles had been inoculated, they were returned to an incubator at 39 ºC for 48 h.

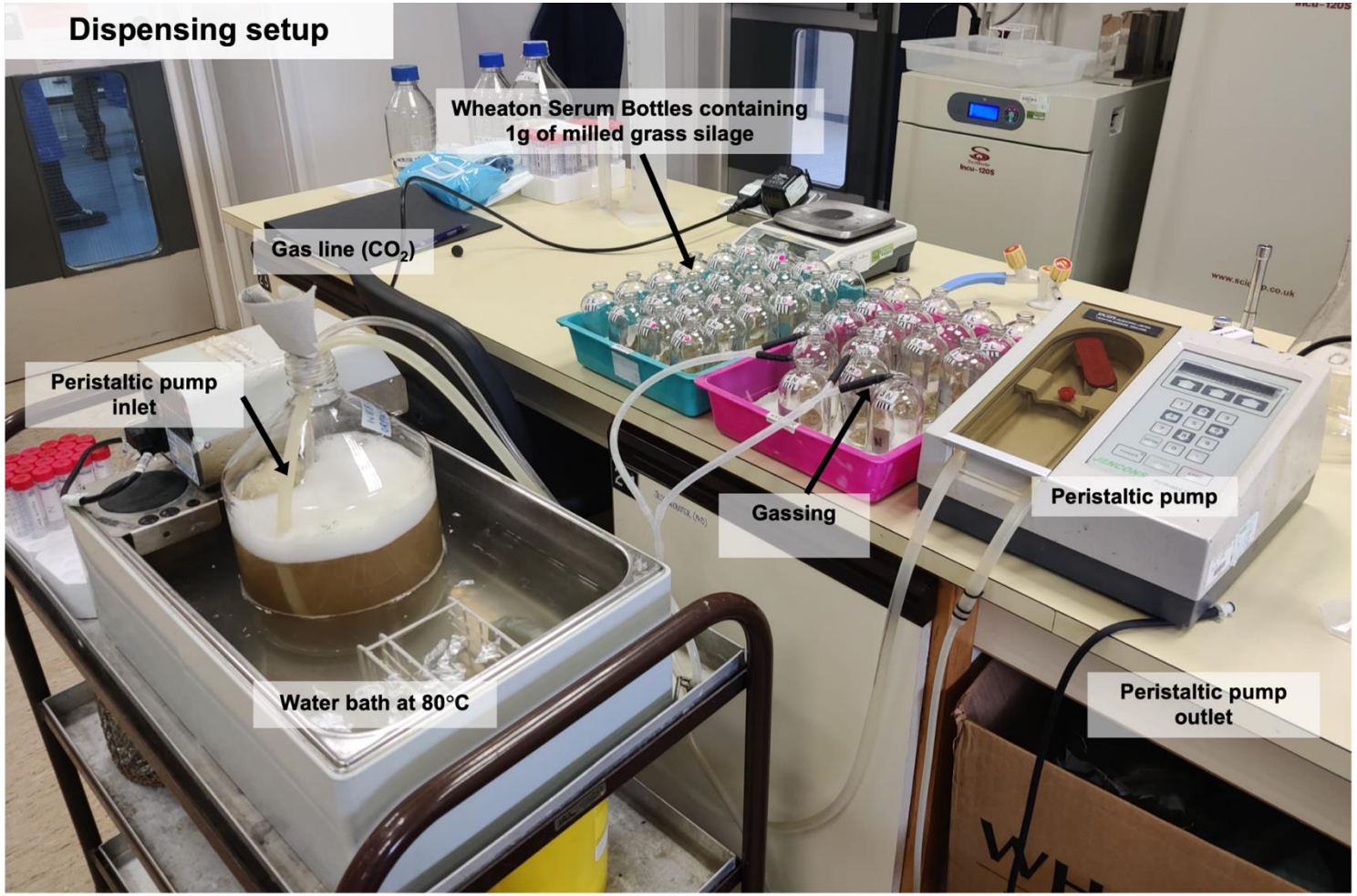


Figure 5-1. Laboratory set up for dispensing buffer medium C via peristaltic pump, into serum bottles already prepared with 1 g of dried and milled grass silage, with gassing needles and sintered thimble (submerged in buffer) attached to the CO_2 line.

Figure 5-2. (Above and centre) Stainless steel 16 G gassing needles attached to plastic “Y” adaptors on the CO_2 gassing line to form an anaerobic environment in the *in-vitro* pilot experiment. (Below) Serum bottles were continually flushed with CO_2 during the dispensing of buffer medium C into the serum bottles.

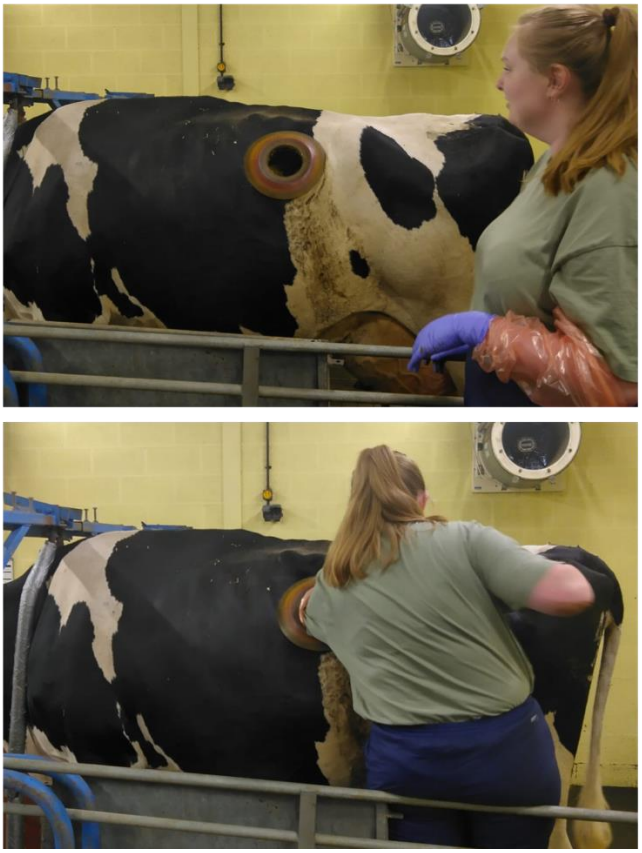


Figure 5-3. (Above) Rumen fistulated Holstein – Friesian dairy cows were restrained in mattress bedded stalls at Harper Adams University for rumen sampling. (Below) The rumen cannula plug was removed by gently pushing in a forward direction and a 250 ml Duran bottle was lowered by gloved hand into the ventral region of the rumen through the 20 cm diameter rumen cannula in order to obtain rumen fluid for experimentation. Cows were restrained for no longer than one hour per rumen fluid sampling session and no more than 2 litres of rumen fluid were removed from an individual cow during sampling.

5.3.1.5. Consecutive batch culture experimental routine

After 48 h of incubation, serum bottles were transferred from the incubator into a 39 ºC water bath. The gas pressure of the bottle was recorded by puncturing the butyl rubber bung with a 20 G needle attached to a gas pressure monitor (Figure 5-5). Under aseptic conditions, a 20 ml luer lock syringe with 20 G needle was used to draw up 10 ml of the culture, which was then injected into a fresh serum bottle containing the following: 90 ml of buffer medium C, 1 g of dried and milled grass silage and the relevant mycotoxin treatment, prepared as described in 5.3.1.2. These newly inoculated batch of serum bottles (generation 2) were returned to the incubator at 39 ºC for 48 h. The older bottles (generation 1) were uncapped, and the pH of the culture recorded as described in Chapter 2. The culture was then dispensed into three sample tubes, comprised of an archive sample, a sample for VFA analysis and ammonia N, prepared as described in Sections 2.1, and 2.2.

Every 48 h a new inoculation was prepared in a fresh set of serum bottles using 10 ml of the previous culture and carried out under aseptic conditions. A total of 15 generations were initially planned.

5.3.1.6. *In-vitro* fermentation model (IFM) experimental routine

The *in-vitro* fermentation model (IFM) is a batch culture technique that was developed to assess the pattern of gas production and digestibility during an incubation of rumen fluid with a feed source (Mjoun et al., 2016). To assess the effect of the mycotoxin treatment on the pattern of gas production, every five generations 10 ml of the culture from the serum bottles was used to inoculate custom designed 250 ml duran bottles (Figure 5-4 ;Dixon Glass, Kent, United Kingdom) for use in the IFM (Figure 5-5). Duran bottles were filled with 1 g of the milled and dried grass silage described in Section 5.3.1.2, 90 ml of buffer and relevant mycotoxin treatment, and secured into the water bath (at 39 °C) of the IFM. The bottles were then flushed with CO_2 by pressurising to approximately 17 pounds per square inch (PSI) and then releasing. Immediately following release, duran bottle lids were attached to the pressure transducers (Cerabar PMC21, Endress + Hauser AG, Reinach, Switzerland). Following this, 10 ml of inoculum from the generation of serum bottles were injected into the duran bottles through the butyl rubber bungs. Gas production from each bottle was recorded every 5 minutes, over 48 h via the pressure transducers and recorded automatically via a monitor calibrated by the manufacturer to display the gas pressure in PSI (Memograph M RSG45, Endress + Hauser AG, Reinach, Switzerland). After 48 h, duran bottles were removed from the water bath and the pH of the culture was recorded using a benchtop monitor and pH probe (Jenway 3510,

Antylia Scientific, Illinois, United States), that had been calibrated using pH 4 and pH 7 standards before use.

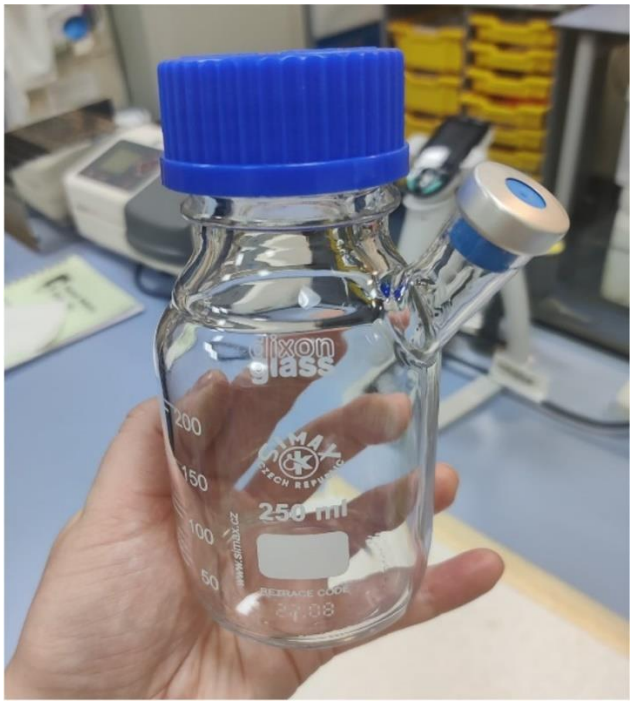


Figure 5-4. Custom-designed duran bottles of 250 ml capacity for use in the *In-vitro* Fermentation model, with an adapted crimp top aluminium neck.

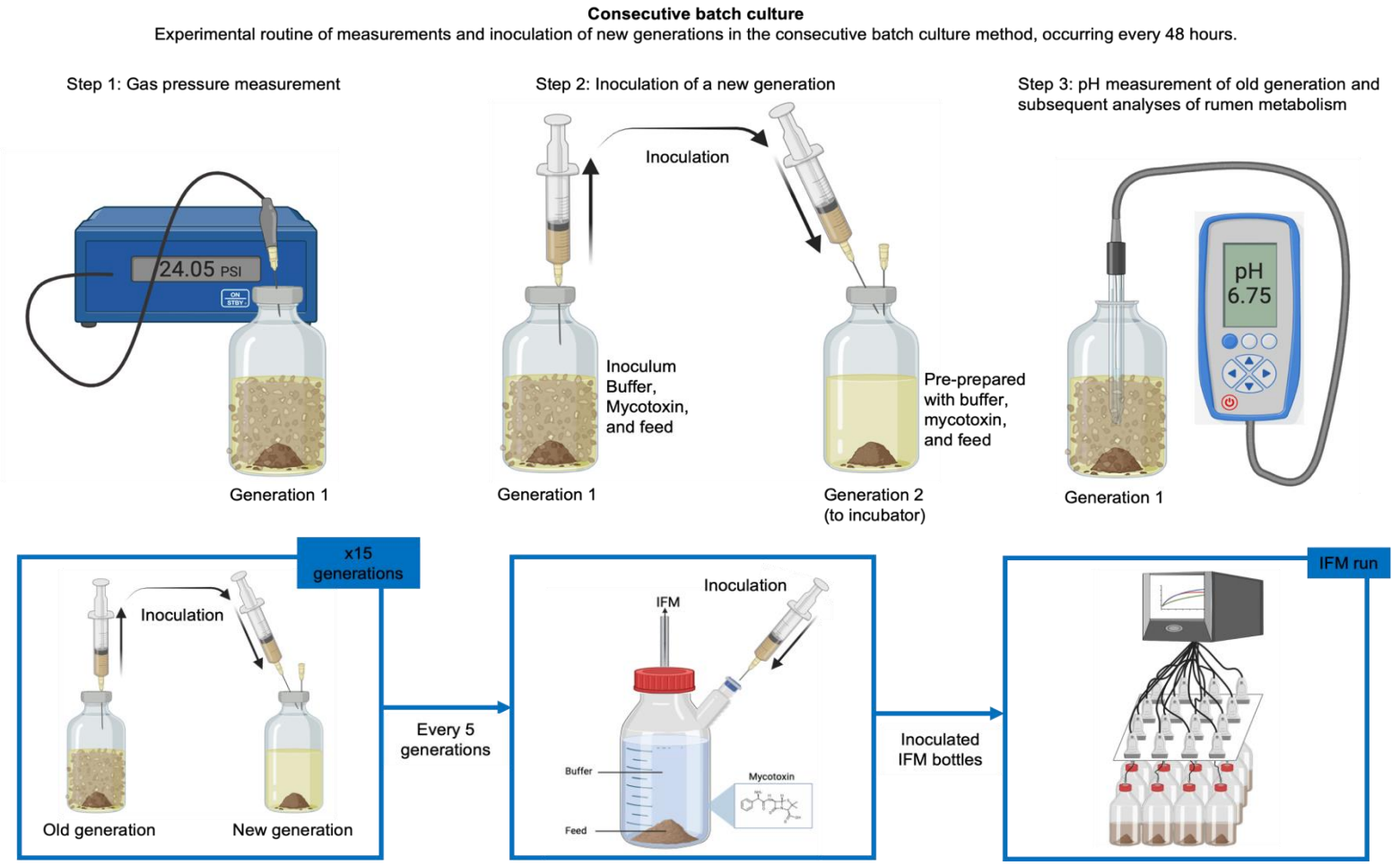


Figure 5-5. Experimental routine outline for the consecutive batch culture method used in the *in-vitro* pilot experiment. Every 48 hours a new generation was inoculated. Every five generations an IFM run was setup and a series of IFM bottles are inoculated alongside a new generation. A total of 15 generations resulted in three IFM runs of 48 hours each, in total. Figure created in Biorender.

5.3.2. Pilot experiment: Results

5.3.2.1. Consecutive batch culture

The consecutive batch culture did not survive past the fourth or fifth generation, with a lack of visual microbial activity on the surface on the inoculum as well as a drop in headspace gas pressure produced to 20-25 PSI from 35-45 PSI at 48 h post-inoculating generation 1 (Figure 5-7). There were no differences observed in pH of the cultures between treatments or levels of inclusion for generations 1 to 5, with a mean of 6.49 (±0.081)

5.3.2.2. *In-vitro* fermentation model

Due to the lack of a fifth generation from the consecutive batch culture, and the drop in headspace pressure from generation 4, no IFM run was carried out for the pilot experiment. It became apparent that the microbial activity was dramatically impacted after the second generation, with a lack of visible activity or biofilm at the surface of the culture (Figure 5-6).

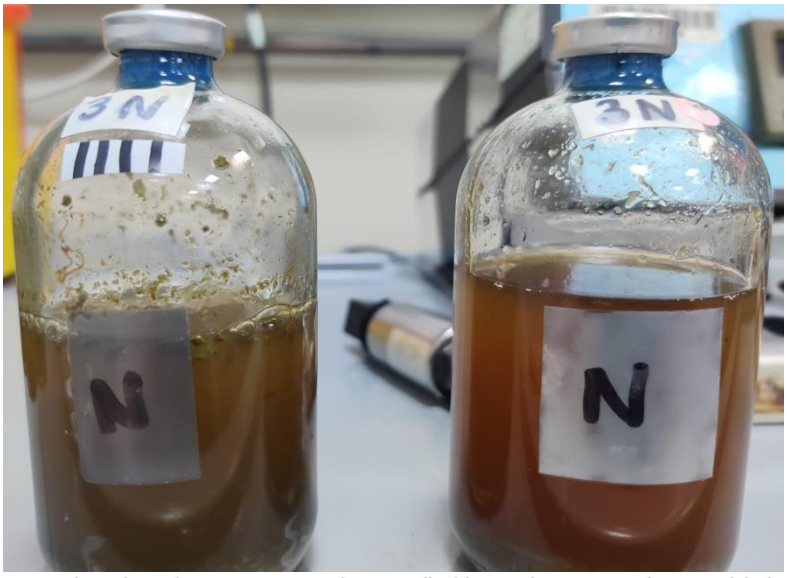


Figure 5-6. Serum bottles from generation 2 (left) and generation 3 (right), in the *in-vitro* pilot experiment. Note the lack of visible microbial film in generation 3 compared with generation 2.

5.3.3. Pilot experiment: Discussion

5.3.3.1. Consecutive batch culture

The consecutive batch culture pilot experiment was unsuccessful due to the early microbial death observed after 3 to 4 generations, in contrast to published work using the same method, which achieved 13 to 15 healthy generations (Davies, 1991; Gascoyne and Theodorou, 1988). It was originally intended that the buffer medium reported by Lowe et al., (1985) (buffer medium B) was to be used in the pilot study, however the numerous constituents of the buffer meant that anaerobic conditions were difficult to maintain. Buffer medium C (Appendix Table 3), as described by Davies, (1991) and adapted from Orpin, (1976) was therefore implemented in the pilot study and all future studies, where buffer constituents could be brought to boil, to purge O_2, and a greater concentration of reducing agents were included compared to buffer medium B.

Despite changes to the buffer in the pilot study to improve the anaerobicity of the cultures, by generation 4 microbial death had occurred. Headspace gas pressure (PSI) for serum bottles that had received treatments where 2 ml of ethanol had been administered, (mycotoxin treatments at “extreme” levels of inclusion and both positive and negative controls), all showed a similar decrease in headspace gas pressures across generations 1-4 (Figure 5-7). Amongst the mycotoxin treated serum bottles, there was a dose response ($P < 0.05$) in headspace gas pressure to the three levels of inclusion, medium, high and extreme, where ethanol addition had been 1.0, 1.5 or 2 ml, respectively. It became apparent that the ethanol used in solubilisation of the mycotoxins, may have impacted on the activity of the rumen inoculum. The bactericidal effect of ethanol is well-documented (Sauerbrei, 2020), however due to the production and utilisation of ethanol by rumen microorganisms themselves (Moomaw and Hungate, 1963) the effect of ethanol was considered to be negligible when planning the study treatments. In contrast, a study by Emery et al., (1959) demonstrated *in-vitro* that the addition of 26.5 mg/L ethanol, suppressed gas production by rumen microbes, and despite metabolism of ethanol occurring in the rumen *in-vivo,* the same was not found *in-vitro*. In comparison to the concentration used by Emery et al., (1959), the pilot study maximum ethanol concentration was 1578 mg/L, almost 60 times higher. Therefore, it was decided that ethanol should be used to solubilise the mycotoxins but then be evaporated prior to incubation for future *in-vitro* experiments.

5.3.4. Pilot experiment: Conclusion for the next experiment

It was not possible to maintain the consecutive batch culture technique beyond four generations. This was most probably due to a very high addition of ethanol that was used to solubilise the mycotoxins to permit an accurate weight to be dispensed into each fermentation tube. Ethanol should therefore be evaporated prior to commencing the *in-vitro* fermentation in future studies, although further work is required to determine that removal of ethanol does not also result in loss of mycotoxins. Additionally with the difficulties experienced during the pilot experiment it was decided that future studies should use a single “generation” IFM incubation (without re-inoculation steps in the CBC method), to reduce the risk of compromising any microbial activity and limiting the fermentation to 48 h only. Ethanol was to be removed before introduction of rumen micro-organisms.

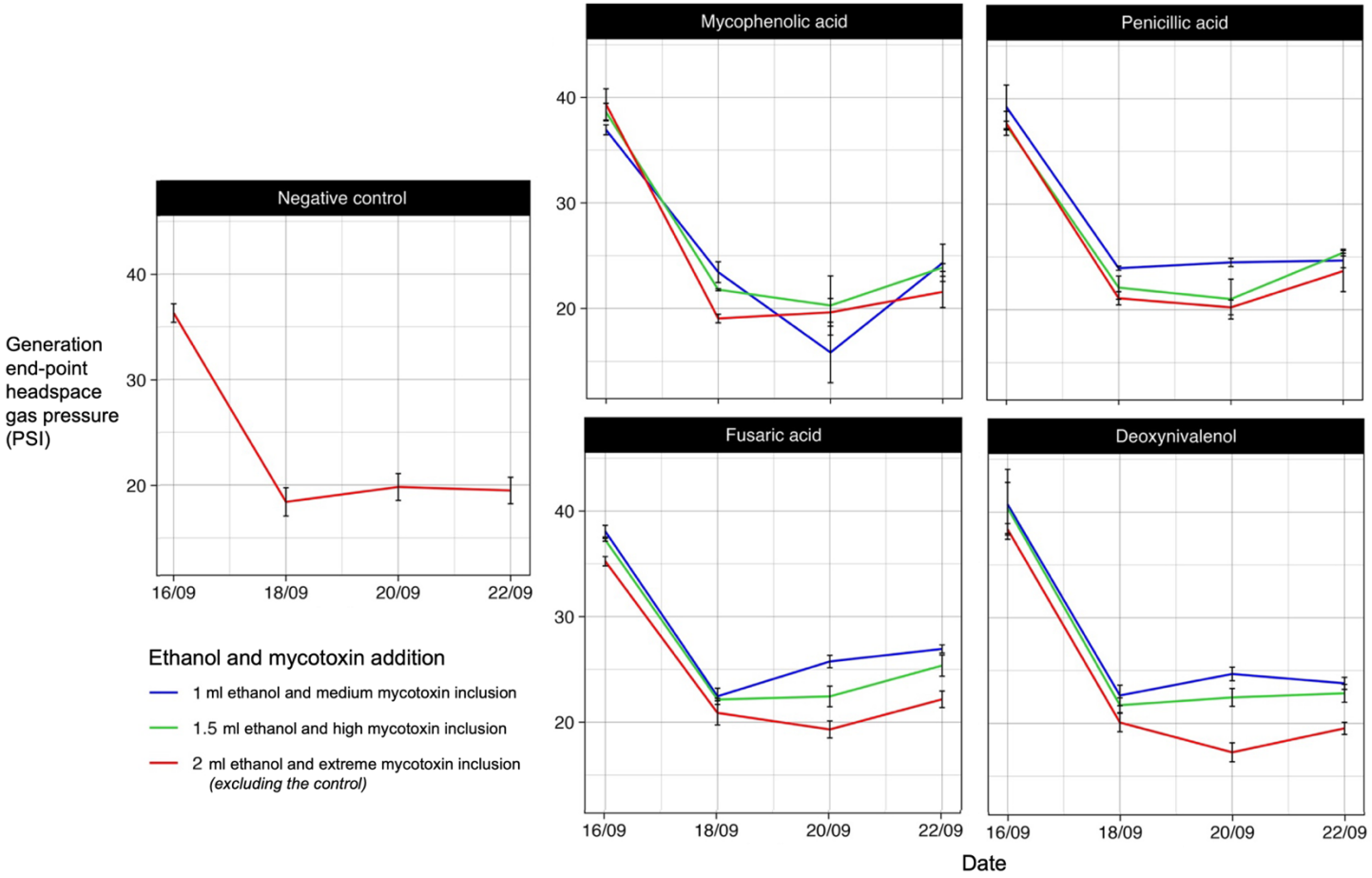


Figure 5-7. Headspace gas pressure (PSI) from consecutive batch culture serum bottles of generation 1 (16/09/2023), 2 (18/09/2023), 3 (20/09/2023) and 4 (22/09/2023) in the *in-vitro* pilot experiment. Bars represent standard error *(n=3).*

5.4. Method development and optimisation of the *in-vitro* experiments to determine the effect of four grass silage mycotoxins on rumen fermentation

Due to the small quantities of mycotoxins required for the *in-vitro* experiments, and lack of solubility in water, solubilisation in ethanol was required to provide an accurate dose rate. To remove ethanol from the solution, prior to the addition of buffer or rumen inoculum, an experiment was undertaken to assess the effect of evaporating off the ethanol, without volatilisation of the mycotoxin. Mycophenolic acid (MPA) (Thermo Fisher Scientific, Massachusetts, United States) was chosen as the mycotoxin to evaluate. Evaporation was carried out by placing the sample on a heat plate set at 60 ºC in a fume cupboard, for 20 minutes. Weight of the sample before and after evaporation was recorded. Between < 0.0001 and 0.0006 g of weight were lost following evaporation. The standard deviation in weights recorded ranged from < 0.0001 – 0.0005.

All other mycotoxins used in the *in-vitro* studies, have a melting point above 83 °C (https://www.ncbi.nlm.nih.gov/, 2024) and so it is unlikely that any volatilisation of the other mycotoxins would occur. In conclusion the process of evaporation, was successful in removing ethanol, whilst leaving the mycotoxin behind.

5.5. *In-vitro* fermentation model (IFM) experiments to determine the effect of four grass silage mycotoxins at three levels of inclusion, or in combination with one another, on rumen microbial metabolism

Following the pilot experiment two separate 48 h incubations of a single rumen inoculation via an *in-vitro* fermentation model (IFM) were undertaken to establish the effects of grass silage mycotoxins on rumen microbial metabolism. These were IFM experiments' A and B.

5.5.1. Hypotheses and objectives

The hypotheses for the following studies are outlined in 5.2, and were investigated in the following studies:

IFM Experiment A: To determine the effect of four grass silage mycotoxins at three levels of inclusion on rumen microbial metabolism *in-vitro*.

IFM Experiment B: To determine the effect of grass silage mycotoxins in combination with one another, on rumen microbial metabolism *in-vitro*.

The following materials and methods were applied to both experiments A and B.

5.5.2. IFM Experiments: Materials and methods:

5.5.2.1. Rumen fluid collection and measurements

Approximately three litres total of strained rumen fluid were collected from three Holstein - Friesian dairy cows fitted with rumen fistula, at midday into three 1 L flasks (Thermos®, West Yorkshire, United Kingdom), following the method outlined in Section 5.3.1.4. Cows were fed different diets between the two experiments, and these are detailed in the respective section for both experiment A and B. Rumen fluid pH was recorded within one minute of collection as described in Section 5.3.1.4. Rumen fluid subsamples of 13.5 ml were decanted into 15 ml sample tubes (Sarstedt, Nümbrecht, Germany) already prepared with 1.5 ml of 25 % w/v meta-phosphoric acid (Sigma-Aldrich®, Germany) for VFA (intra-assay % CV: 8.30) and ammonia N analysis (intra-assay % CV: 2.74) as described in Sections 2.1 and 2.2. All sample tubes and three 500 g grab samples of the TMR for each cow, were frozen at -20 °C until subsequent analysis.

5.5.2.2. *In-vitro* fermentation model preparation

The individual mycotoxins (IFM Experiment A) or combinations of mycotoxins (IFM Experiment B) were dissolved in ethanol as described in Section 5.3.1.3 and pipetted into 250 ml duran bottles (fermenters) that had been pre-filled with 0.5 g of dried and milled grass silage (Table 5-2). The bottles were then set on top of a heat plate at 60°C in a fume cupboard for 20 minutes, to evaporate off the ethanol as described in Section 5.4. Following evaporation, fermenters were sealed with GL 45 screw lids adapted with an inlet/outlet for the IFM pressure transducer attachments. Fermenters were slotted into the IFM water bath at 39°C at least 2 hours before addition of the buffer and rumen inoculum. Fermenter vessels were flushed with CO_2 (BOC, Surrey, United Kingdom) through the inlet/outlet, and left under pressure at approximately 16 PSI (~1.3 PSI above atmospheric pressure).

Rumen fluid collected from the three cows as described in Section 5.3.1.4, was mixed proportionally (1:1:1) in the laboratory to form a combined inoculum and the pH was recorded. Buffer medium C was formulated according to the method outlined in Section 5.3.1.2. After gassing the buffer for 1.5 h under CO_2, the combined rumen fluid inoculum was added to the buffer in a ratio of 80:20 buffer to rumen fluid and pH recorded. Using a peristaltic pump (Jencons Perimatic, Bedfordshire, United Kingdom) 100 ml of buffer and rumen fluid inoculum was added to the each of the bottles. Pressure was released from the vessels through the inlet/outlet, to approximately 14.7 PSI (atmospheric pressure) and then attached to the IFM pressure transducers to measure gas production over 48 h (Figure 5-8).

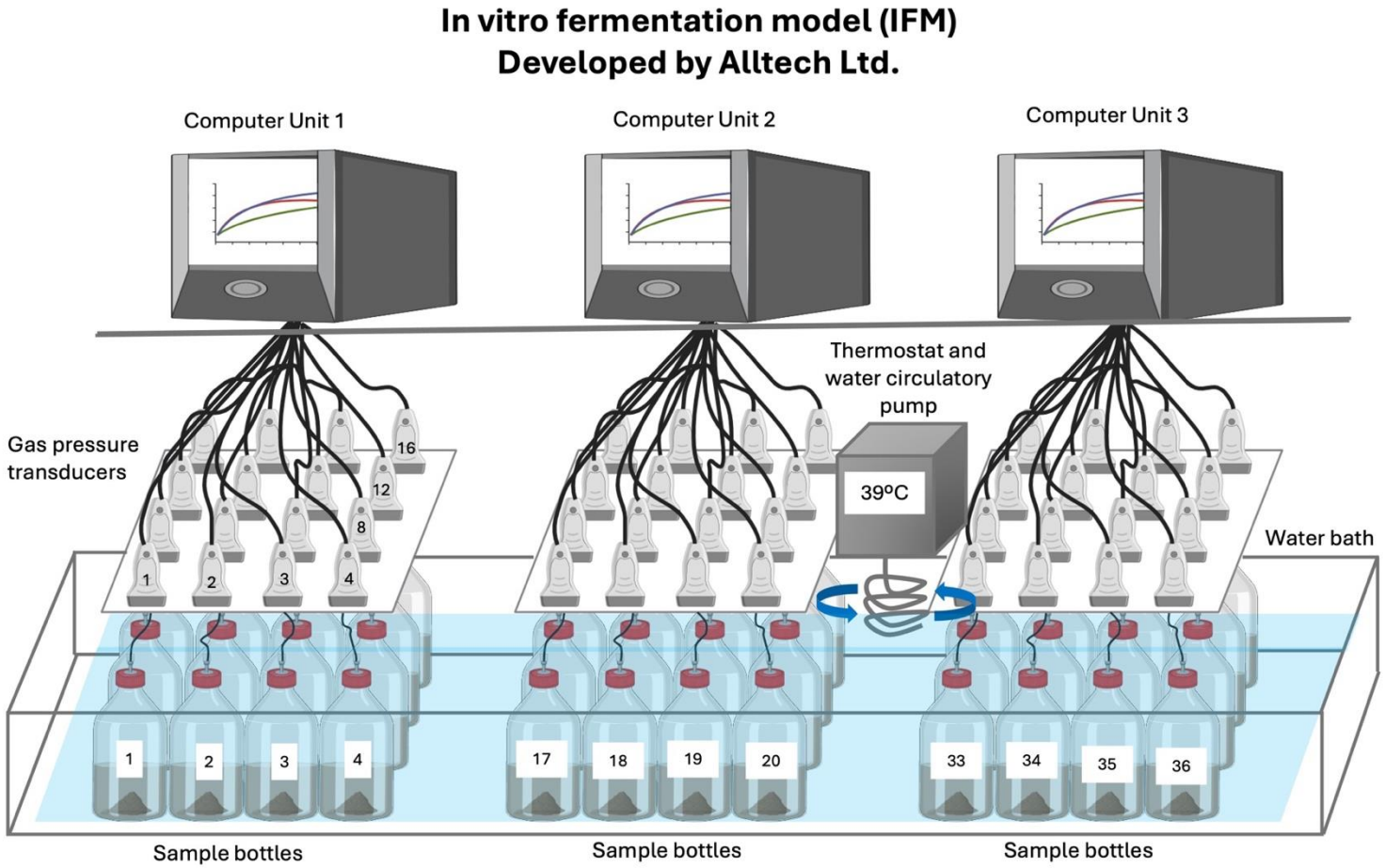


Figure 5-8. The *In-vitro* fermentation model set up for IFM experiments' A and B. Fermentation vessels containing dried and milled grass silage, mycotoxin treatment and rumen fluid and buffer inoculum are attached to the pressure transducers via air-tight gas lines from the lid of the fermentation vessels. Vessels are placed in a water bath at 39°C for 48 h. Gas production via pressure increase (PSI) is monitored via the IFM computer units with data recorded every five minutes for the duration of the fermentation.

Table 5-2. Silage chemistry and mycotoxin profile of the grass silage used in the *in-vitro* studies

Silage chemistry (g/kg DM)	Combined[1]
Dry matter	282
Crude protein	97.6
Neutral detergent fibre	460
Ammonia N (% Total N)	8.16
pH	3.85
Lactic acid	98.3
Acetic acid	35.3
Propionic acid	< 0.591
Butyric acid	< 0.131
Isobutyric acid	< 0.063
Valeric acid	< 0.010
Isovaleric acid	< 0.133
Hexanoic acid	< 0.010
Heptanoic acid	< 0.010
Ethanol	3.55
Propan-1-ol	< 0.010
Propane-1,2-diol	17.2
Lactic acid : acetic acid	2.75
Silage mycotoxins (µg/kg DM)	
Risk equivalent quantity	1507
Ergocryptin(in)e	2.36
Fumonisin B_1	134
Fumonisin B_2	43.0
Fusaric acid	19.1
Penicillic acid	2006

[1]Combined. The grass silage used was a 1:1 combined sample of 1 kg collected from the top section of the clamp as described in Chapter 3, and 1 kg collected from the mid section of the clamp.

5.5.2.3. *In-vitro* fermentation model measurements

After 48 h, the fermenters were removed from the IFM water bath, and 30 ml of gas was drawn into a 60 ml luer lock syringe for methane determination by GC. Remaining gas pressure was released, and the pH of the culture was recorded immediately. A one ml subsample of culture was pipetted into 1.5 ml Eppendorf tubes for subsequent VFA analysis, as described in Chapter 2. Approximately 15 ml of culture was then pipetted into 15 ml falcon tubes for ammonia N determination of the culture as described in Section 2.2. The gas pressure profile (PSI) in 5 minute intervals for each fermenter over the 48 h fermentation period was downloaded from the IFM computer console. The gas pressure for the end time of the run was

adjusted for starting pressure calculated as a mean of three blank bottles that were included in the run.

5.5.3. Determination of methane headspace concentration

Gas sampled into luer lock 60 ml syringes were injected in duplicate (approximately 15 ml of gas per replicate; intra-assay % CV: 8.79) into the manual inlet of the gas chromatography machine (GC, Agilent 7890 GC System, Agilent Technologies™, California, United States) and using a Porapak QS Packed GC Column, 80-100 mesh, with a length of 1.82 m and diameter of 2.1 mm (Agilent Technologies™, California, United States). The parameters of the GC were: front inlet temperature: 100°C, pressure: 14.652 psi, flow: 4ml/min, heater 300°C, split ratio: 4:1, split flow: 16 ml/min and total flow 23 ml/min. The GC was calibrated with external standard preparations of 5 to 100 ppm methane in air, increasing by an interval of 5 ppm. All measurements were carried out in duplicate. A standard of 350 ppm methane (BOC, Woking, United Kingdom) was injected into the GC front inlet before the samples to check the calibration was within 97 % of the standard. Quantification was calculated as the area of the peak of the sample compared with the area of the peak of the standard.

5.5.4. Statistical analysis

All statistical analysis was carried out in R (R Core Team, V 4.1.3., Vienna, Austria), using the packages `dplyr`, and `ggplot2` for data manipulation and visualisation, respectively. Analysis of variance was carried out with the following models:

Experiment A:

$$Y_{ij} = \mu + T_i + D_j + (TD)_{ij} + \varepsilon_{ij}$$

Experiment B:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$

Where, Y_{ij} = dependent variable; μ = overall mean; T_i = main effect of the i-th treatment (mycotoxin addition), D_j = main effect of the j-th dose (level of inclusion at medium, high or extreme); $(TD)_{ij}$ = interaction effect between treatment (T) and dose (D); and ε_{ij}= residual error.

Post-hoc analyses was carried out using Dunnett's test in the `DescTools` package (Dunnett, 1955). Significance was considered at $P < 0.05$ and a trend considered at $P > 0.05$ but < 0.10.

5.6. IFM Experiment A: Determining the effect of four grass silage mycotoxins at three levels of inclusion on rumen microbial metabolism *in-vitro*.

The effects of four grass silage mycotoxins at three levels of inclusion on the rumen microbial metabolism was determined via a 48 h fermentation in the IFM, as outlined in Table 5-1. Rumen fluid was collected as described in Section 5.3.1.4, from Holstein-Friesian cows (multiparous, 188 ± 128 DIM) fed the following diet: a 65:35 ratio of forage to concentrates (where the forage was 210 g/kg DM of grass silage, 442 g/kg DM of maize silage) formulated to provide a crude protein content of 149 g/kg DM, a neutral detergent fibre content of 364 g/kg DM, and an ME content of 12.1 MJ/kg DM.

5.6.1. IFM Experiment A: Treatments

The treatments for the *in-vitro* experiment A are outlined in Table 5-1, in Section 5.3.1.1.

5.6.2. IFM Experiment A: Results

5.6.2.1. Rumen fluid measurements and gas production

Mean rumen fluid pH was 6.27 (± 0.294), and the resultant combined rumen fluid and buffer (inoculum) had a pH of 7.26. *In-vitro* fermentation vessels 23, 37 and 43 were removed from the dataset due to suspected air leak compromising the gas pressure and anaerobicity of the culture (Figure 5-9).

There was no effect of treatment (Trt; P = 0.685) or level of inclusion (Dose; P = 0.978) on gas pressure increase for any of the mycotoxin treated cultures (mean: 9.07 PSI ± 0.333; Table 5-3) in comparison to the control (mean: 8.06 PSI). There was also no interaction between the treatment and level of inclusion on the gas pressure increase (Trt x Dose; P = 0.974). Headspace methane (kg/m^3) produced was affected by treatment (P = 0.003) and level of inclusion (P = 0.001) but *post-hoc* analysis revealed that no differences were observed between mean concentrations for the mycotoxin treated cultures (mean: 0.032 kg/m^3 ± 0.005) and the control (mean: 0.033 kg/m^3 ; Table 5-3). There was also no interaction effect observed (P = 0.271).

5.6.2.2. Culture fluid pH, ammonia and VFA concentration

There was no effect of mycotoxin treatment (P = 0.351), level of inclusion (P = 0.385) or an interaction (P = 0.747) on the concentration of ammonia N (mg/L) in the rumen fermentation cultures, in comparison to the control (mean: 138 mg/L ± 19.5; Table 5-3). Similarly, there was no effect of mycotoxin treatment (P = 0.814) or level of inclusion (P = 0.092) on the pH of the

rumen fermentation culture in comparison to the control (mean: 6.95 ± 0.022; Table 5-3). There was also no interaction between treatment and level of inclusion (P = 0.919).

There was an effect of mycotoxin treatment on propionate (P = 0.003), butyrate (P = 0.003), and isovalerate concentration (P = 0.029) of the rumen fermentation cultures (Table 5-3) however *post-hoc* analysis revealed no difference in means between the mycotoxin treated cultures in comparison to the control. There was an effect of level of inclusion on the total VFA concentration (P = 0.007), acetate (P = 0.004), butyrate (P = 0.001), and valerate (0.014) concentrations, however *post-hoc* analyses again, revealed no difference in means between the mycotoxin treated cultures and the control. There were no effects of an interaction between mycotoxin treatment and level of inclusion observed on concentrations of VFAs in the rumen fermentation cultures (all P > 0.05).

5.6.2.3. Culture VFA proportions

There was an effect of treatment on proportions of acetate (P = 0.004), propionate (P < 0.001), butyrate (P = 0.026), isobutyrate (P < 0.001) and isovalerate (P = 0.006) as a percentage of the total VFAs, however *post-hoc* analyses demonstrated no difference between means of the control fermentation culture and the mycotoxin treated fermentation cultures (Table 5-3). There was also an effect of level of inclusion on the proportion of acetate (P = 0.003), propionate (P < 0.001), isobutyrate (P < 0.001), valerate (P = 0.001) and isovalerate (P < 0.001) as a percentage of total VFAs. *Post-hoc* analyses demonstrated no difference between the means of control and mycotoxins at the three levels of inclusion. There were no interactions between the mycotoxin treatment and the level of inclusion on the VFAs as a percentage of total VFAs (all P > 0.05).

There was an effect of treatment on the acetate to propionate ratio (P = 0.009) and level of inclusion (P = 0.001) however, no difference was found between the control fermentation cultures (mean: 2.40) and the mycotoxin treated fermentation cultures (mean: 2.38 ± 0.353; Table 5-3). There was also an effect of treatment on the acetate + butyrate to propionate ratio (P = 0.006) and level of inclusion (P < 0.001) but again, no difference was found between the means for the control (mean: 2.94) and the mycotoxin treated cultures (mean: 2.92 ± 0.375). Similarly, there were no interactions between the mycotoxin treatment and the level of inclusion for either the acetate : propionate ratio (P = 0.702) or the acetate + butyrate : propionate ratio (P = 0.644).

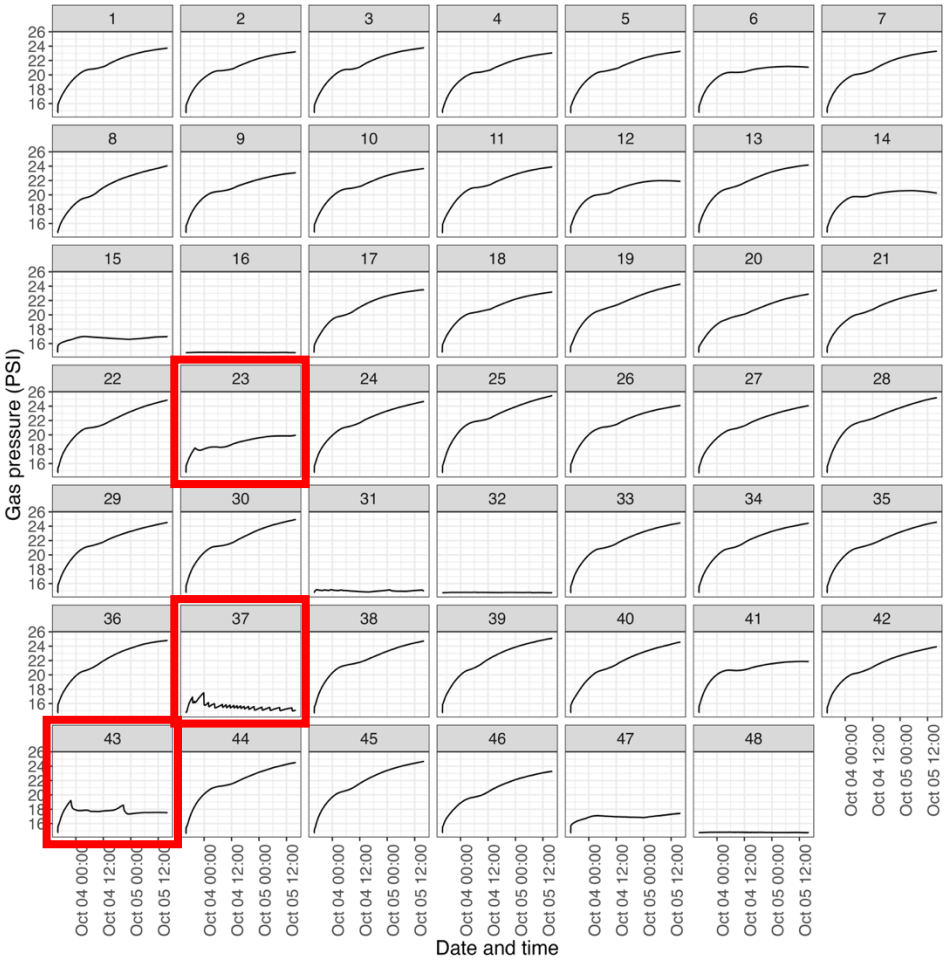


Figure 5-9. Pattern of gas production (gas pressure, PSI) for each IFM fermenter vessel over the 48 h incubation, after inoculation with either medium, high or extreme concentrations of four grass silage mycotoxins in the *in-vitro* A experiment. Bottles numbered 23, 37 and 43 (outlined in red) were removed from subsequent analysis. Bottles numbered 16, 32, and 48 represent empty bottles (blanks), and bottles numbered 15, 31 and 47 represent a control for the buffer medium C, where bottles contained buffer medium C (no rumen inoculum) and substrate only.

Table 5-3. Gas production, ammonia, pH and volatile fatty acid concentrations (VFA mM) of the IFM bottle cultures, 48 h after inoculation with either medium, high or extreme concentrations of four grass silage mycotoxins in the *in-vitro* A experiment.

In-vitro A	Control	Medium				High				Extreme					Significance		
		MPA[3]	PEN[4]	FUS[5]	DON[6]	MPA	PEN	FUS	DON	MPA	PEN	FUS	DON	s.e.d.[7]	Trt[8]	Dose[9]	Trt x Dose
Gas pressure increase (PSI)*	8.06	9.14	8.82	9.44	9.13	8.84	8.59	9.67	9.23	9.44	8.79	8.71	9.09	2.671	0.685	0.978	0.974
Headspace methane (kg/m^3)	0.033	0.030	0.030	0.032	0.027	0.027	0.029	0.035	0.027	0.029	0.040	0.043	0.034	0.0108	0.003	0.001	0.271
Ammonia N (mg/L)	130	152	160	169	104	154	159	143	119	126	121	134	125	82.7	0.351	0.385	0.747
pH	6.93	6.96	6.98	6.97	6.96	6.97	6.95	6.93	6.96	6.94	6.93	6.92	6.91	0.104	0.814	0.092	0.919
VFAs (mM)																	
Total VFA's	38.4	38.1	37.8	35.0	38.2	38.1	37.1	39.7	39.1	39.7	39.6	44.8	43.2	7.57	0.544	0.007	0.336
Acetate	21.8	20.2	20.5	19.9	21.2	20.2	19.5	23.1	21.7	21.9	22.5	28.1	26.7	6.78	0.172	0.004	0.484
Propionate	8.97	10.0	9.45	8.08	9.39	10.1	9.71	8.77	9.54	9.71	8.53	9.03	8.83	1.37	0.003	0.319	0.412
Butyrate	4.93	5.18	4.94	4.20	4.87	5.27	5.30	4.61	5.03	5.55	5.21	5.30	5.18	0.686	0.003	0.001	0.270
Iso-butyrate	0.006	0.066	0.025	0.101	0.018	0.044	0.023	0.048	0.035	0.057	0.032	0.034	0.021	0.0734	0.077	0.394	0.528
Valerate	1.20	1.15	1.45	0.992	1.36	1.11	1.17	1.35	1.36	1.09	1.08	1.06	1.15	0.343	0.521	0.014	0.344
Iso-valerate	0.880	0.961	0.937	0.822	0.908	0.949	0.916	0.870	0.941	0.908	0.906	0.885	0.862	0.1141	0.029	0.629	0.529
VFA as a % of total VFA																	
Acetate	56.7	53.1	54.1	54.8	55.4	52.9	54.3	57.2	58.5	55.1	56.8	61.5	60.7	5.82	0.004	0.003	0.806
Propionate	23.5	26.3	25.0	25.1	24.5	26.4	25.1	23.4	22.9	24.5	23.0	20.6	21.0	3.22	<0.001	<0.001	0.761
Butyrate	13.1	13.6	13.1	12.7	12.7	13.8	13.7	12.3	12.0	14.0	13.5	12.3	12.4	2.28	0.026	0.990	0.948
Iso-butyrate	1.34	1.50	1.49	1.39	1.39	1.45	1.44	1.34	1.28	1.36	1.30	1.19	1.20	0.161	<0.001	<0.001	0.968
Valerate	3.16	3.03	3.81	3.61	3.58	2.92	3.05	3.53	3.07	2.76	3.00	2.45	2.74	0.956	0.440	0.001	0.388
Iso-valerate	2.29	2.52	2.48	2.39	2.38	2.49	2.43	2.25	2.22	2.29	2.31	2.04	2.05	0.314	0.006	<0.001	0.948
Ac : Prop[1]	2.40	2.05	2.17	2.34	2.28	2.02	2.02	2.53	2.28	2.27	2.47	3.06	3.04	0.699	0.009	0.001	0.702
Ac + But : Prop[2]	2.94	2.57	2.69	2.84	2.80	2.55	2.56	3.04	2.81	2.85	3.06	3.65	3.63	0.680	0.006	< 0.001	0.644

* Corrected for starting pressure of 14.76 PSI

[1]Ac : Prop : acetate to propionate ratio; [2] Ac + But : Prop: acetate + butyrate to propionate ratio;

[3]MPA: mycophenolic acid; [4]PEN: penicillic acid; [5]FUS: fusaric acid; [6]DON: deoxynivalenol;

[7]s.e.d.: standard error of the difference between means; [8]Trt: Mycotoxin treatment; [9]Dose: Mycotoxin dosage/ level of inclusion (Medium, high or extreme).

5.7. IFM Experiment B: Investigating the effect of grass silage mycotoxins in combination on rumen microbial metabolism.

The effects of four grass silage mycotoxins at the high level of inclusion in combination with one another on the rumen microbial metabolism was determined via a 48 h fermentation in the IFM, as outlined in Table 5-1. Rumen fluid was collected as described in Section 5.3.1.4, from Holstein-Friesian cows (multiparous, 196 ± 177 DIM) fed the following diet: a 63:37 ratio of forage to concentrates (where the forage was 160 g/kg DM of grass silage, 405 g/kg DM of maize silage, and 62.7 g/kg of lucerne silage) formulated to provide a crude protein content of 162 g/kg DM, a neutral detergent fibre content of 346 g/kg DM, yielding 47.5 kg milk day^{-1}.

5.7.1. IFM Experiment B: Treatments

The treatments for the *in-vitro* experiment B, are outlined in Table 5-4.

Table 5-4. Treatment outline for the *in-vitro* fermentation model experiment B, where a rumen fluid culture was subject to a 48 h incubation with either mycophenolic acid alone (MPA) or in combination with penicillic acid (PEN), fusaric acid (FUS) or deoxynivalenol (DON).

Treatment code	Treatment details	Level of inclusion/dose	Mycotoxin µg/g DM	Ethanol µg/g DM
CONTROL	100% Ethanol	-	-	1.50
MPA ONLY	Mycophenolic acid	High	5.00	1.50
PEN ONLY	Penicillic acid	High	4.00	1.50
FUS ONLY	Fusaric acid	High	4.50	1.50
DON ONLY	Deoxynivalenol	High	2.25	1.50
MPA x PEN	Mycophenolic acid x Penicillic acid	High	5.00, 4.00	1.50
MPA x FUS	Mycophenolic acid x Fusaric acid	High	5.00, 4.50	1.50
MPA x DON	Mycophenolic acid x Deoxynivalenol	High	5.00, 2.25	1.50
PEN x FUS	Penicillic acid x Fusaric acid	High	4.00, 4.50	1.50
PEN x DON	Penicillic acid x Deoxynivalenol	High	4.00, 2.25	1.50
FUS x DON	Fusaric acid x Deoxynivalenol	High	4.50, 2.25	1.50
FIELD MIX (FMIX)	Penicillic acid x Fusaric acid x Deoxynivalenol	High	4.00, 4.50, 2.25	1.50
ALTOGETHER (ALT)	Penicillic acid x Fusaric acid x Deoxynivalenol x Mycophenolic acid	High	4.00, 4.50, 2.25, 5.00	1.50

5.7.2. IFM Experiment B: Results

5.7.2.1. Gas production, methane concentration, and pH in cultures when MPA, PEN, FUS, or DON, was administered alone at a medium level of inclusion

There was no effect of treatment on gas pressure increase (P = 0.076) with a mean of 7.00 PSI ± 0.636, or headspace methane (P =0.595) with a mean of 0.031 kg/m^3 ± 0.0011 at 48 h post inoculation. There was also no effect of treatment (P = 0.975) on the pH with a mean pH of 6.84 ± 0.01 after a 48 h incubation.

5.7.2.2. VFA concentrations in cultures when MPA, PEN, FUS, or DON, was administered alone at a medium level of inclusion

There was no effect of mycotoxin treatment on total VFAs (P = 0.263), acetate (P = 0.272), propionate (P = 0.234) or butyrate (P = 0.283) concentration of the rumen fermentation cultures, with means of 39.8 mM ± 2.84, 19.7 mM ± 1.34, 11.5 mM ± 0.921 and 5.59 mM ± 0.411, respectively. Additionally, there was no effect of treatment on isobutyrate (P = 0.441), valerate (P = 0.152) and isovalerate (P = 0.276) concentrations in the fermentation cultures after a 48 h incubation, with means of 0.738 mM ± 0.0458, 0.974 mM ± 0.0757, and 1.30 mM ± 0.087, respectively.

5.7.2.3. VFA proportions in the cultures when MPA, PEN, FUS, or DON, was administered alone at a medium level of inclusion

There was no effect of mycotoxin treatment on acetate (P = 0.323), propionate (P = 0.122) or butyrate (P = 0.595) as a percentage of total VFAs in the rumen fermentation cultures, with means of 49.5 % ± 0.483, 28.9 % ± 0.474 and 14.2 % ± 0.400, respectively. Additionally, there was no effect of treatment on isobutyrate (P = 0.476), valerate (P = 0.161) and isovalerate (P = 0.085) as a percentage of total VFAs in the rumen fermentation cultures after a 48 h incubation, with means of 1.86 % ± 0.626, 2.45 % ± 0.094, and 3.25 % ± 0.059, respectively.

There was also no effect of treatment on acetate to propionate ratio (P = 0.194), with a mean of 1.72 ± 0.048, or on acetate + butyrate to propionate ratio (P = 0.159) with a mean ratio of 2.20 ± 0.051.

5.7.2.4. Gas production, methane concentration and pH in cultures when MPA, PEN, FUS or DON was administered in combination with one another

There was no effect of treatment observed for the gas pressure increase (P = 0.454) with a mean of 7.65 PSI ± 0.149. There was an effect of mycotoxin treatment on headspace methane concentration (P = 0.041) however, *post-hoc* analyses revealed no difference between the control and any of the mycotoxin combinations; but that the difference was between ALT and DON x FUS and DON x MPA treated cultures after a 48 h incubation. The mean value for headspace methane was 0.033 kg/m^3 ± 0.0016. There was no effect of treatment on pH (P = 0.901) with a mean value of 6.82 ± 0.020.

5.7.2.5. VFA concentrations in the cultures when MPA, PEN, FUS or DON was administered in combination with one another

There was a trend (P = 0.069) for PEN x MPA, and PEN x DON treated cultures to demonstrate a lower concentration of total VFAs in comparison to the control, with means of 3.58 mM, and 44.1 mM, respectively. There was no effect of treatment on acetate concentrations in the fermentation cultures with a mean of 19.2 mM ± 1.25. There was, however, an effect of mycotoxin treatment on propionate (P = 0.048) and butyrate (P = 0.015) concentrations in the fermentation cultures after a 48 h incubation. For propionate concentrations, PEN x DON and PEN x MPA demonstrated means of 10.0 and 10.3 mM respectively, lower in comparison to the mean of 13.0 mM in the control treated cultures. In concentrations of butyrate, cultures treated with PEN x DON, PEN x FUS and PEN x MPA demonstrated lower means of 4.79 mM, 5.02 mM and 4.82 mM, respectively, in comparison with the control (6.22 mM). There was no effect of treatment on isobutyrate, but there was a trend demonstrated in valerate concentrations of the fermentation cultures (P = 0.054) where PEN x DON and PEN x MPA treated cultures demonstrated lower means of 0.862 mM and 0.846 mM respectively, in comparison to the control valerate concentration with a mean of 1.07 mM. There was an effect of treatment on isovalerate concentrations (P = 0.049) where the PEN x DON treated cultures, resulted in a lower mean isovalerate concentration of 1.17 mM in comparison to the control, of 1.42 mM

5.7.2.6. VFA proportions in the cultures when MPA, PEN, FUS or DON was administered in combination with one another

There was no effect of mycotoxin treatment on acetate, propionate, or butyrate as a percentage of total VFAs with means of 50.2 % ± 0.670, 28.7 % ± 0.506 and 13.6 % ± 0.243, respectively. There was an effect of treatment on isobutyrate as a percentage of total VFAs

(P = 0.030) however *post-hoc* analyses revealed that these differences were between PEN x FUS and PEN x MPA treated cultures, and not between mycotoxin combination treated cultures and the control. There was no effect of mycotoxin treatment on valerate (P = 0.374), or isovalerate (P = 0.417) as a percentage of total VFAs with mean values of 2.38 % ± 0.037 and 3.23 % ± 0.044, respectively.

There was no effect of mycotoxin treatment on either ratio of acetate to propionate (P = 0.374) or acetate + butyrate to propionate (P = 0.417) with mean values of 1.75 ± 0.053, and 2.22 ± 0.054 respectively.

Table 5-5. Fermentation parameters for the rumen fluid culture after a 48 h incubation with either mycophenolic acid (MPA), penicillic acid (PEN), fusaric acid (FUS) or deoxynivalenol (DON) in *in-vitro* experiment B.

In-vitro B	Control	MPA[3]	PEN[4]	FUS[5]	DON[6]	s.e.d.[7]	Significance Trt[8]
Gas pressure increase (PSI)*	7.31	6.44	7.56	6.19	7.49	0.881	0.076
Headspace methane (kg/m^3)	0.031	0.031	0.033	0.032	0.030	0.0025	0.595
pH	6.85	6.84	6.82	6.86	6.85	0.099	0.975
VFAs (mM)							
Total VFAs	44.1	40.7	36.5	38.4	39.5	5.03	0.263
Acetate	21.6	20.1	18.0	19.0	19.9	2.39	0.272
Propionate	13.0	11.8	10.6	11.2	11.1	1.61	0.234
Butyrate	6.22	5.73	5.16	5.34	5.48	0.747	0.283
Iso-butyrate	0.800	0.767	0.690	0.732	0.701	0.1007	0.441
Valerate	1.069	0.965	0.890	0.914	1.030	0.1183	0.152
Iso-valerate	1.42	1.34	1.21	1.22	1.29	0.158	0.276
VFA as a % of total VFAs							
Acetate	49.0	50.3	49.6	49.3	49.5	0.95	0.323
Propionate	29.5	28.2	28.8	28.9	29.1	0.73	0.122
Butyrate	14.1	13.9	14.0	14.1	13.9	0.29	0.595
Iso-butyrate	1.80	1.78	1.90	1.90	1.91	0.143	0.476
Valerate	2.42	2.61	2.39	2.44	2.38	0.154	0.161
Iso-valerate	3.21	3.26	3.31	3.31	3.18	0.087	0.085
Ac : Prop[1]	1.66	1.79	1.73	1.71	1.70	0.078	0.194
Ac + But : Prop[2]	2.14	2.28	2.21	2.19	2.18	0.084	0.159

*Gas pressure corrected for initial pressure of 16.41 PSI
[1]Ac : Prop = acetate : propionate ratio, [2]Ac + But : Prop = acetate + butyrate : propionate ratio
[2]PEN = penicillic acid, [3]MPA = mycophenolic acid, [5]FUS = fusaric acid, [6]DON = deoxynivalenol
[7]s.e.d. = standard error of the difference between means, [8]Trt = treatment.

Table 5-6. Fermentation parameters for the rumen fluid culture after a 48 h incubation with either dual combinations of penicillic acid (PEN), fusaric acid (FUS), deoxynivalenol (DON) or mycophenolic acid (MPA), or the field mix (FMIX) and altogether combinations (ALT) in the *in-vitro* experiment B.

In-vitro B	CON	Dual combinations						Multiple combinations		s.e.d.[9]	Significance
		PEN[3] x DON[4]	PEN x FUS[5]	PEN x MPA[6]	DON[6] x FUS	DON x MPA	MPA x FUS	FMIX[7]	ALT[8]		Trt[10]
Gas pressure increase (PSI)*	7.31	7.62	7.35	7.64	7.58	7.49	7.30	7.58	8.97	1.507	0.454
Headspace methane (kg/m^3)	0.031	0.033	0.032	0.032	0.035	0.035	0.032	0.033	0.030	0.0031	0.041
pH	6.85	6.81	6.85	6.82	6.83	6.81	6.80	6.82	6.80	0.092	0.901
VFAs (mM)											
Total VFAs	44.1	35.8	36.9	35.8	39.3	39.0	38.1	37.1	38.1	6.41	0.069
Acetate	21.6	18.3	18.5	17.9	19.7	19.7	18.9	18.7	19.2	3.13	0.157
Propionate	13.0	10.0	10.7	10.3	11.4	11.1	11.1	10.7	11.0	2.07	0.048
Butyrate	6.22	4.79	5.02	4.82	5.33	5.26	5.25	4.99	5.11	0.896	0.015
Iso-butyrate	0.800	0.663	0.644	0.731	0.701	0.787	0.707	0.705	0.720	0.1418	0.236
Valerate	1.07	0.862	0.885	0.846	0.933	0.899	0.905	0.868	0.935	0.1514	0.054
Iso-valerate	1.42	1.17	1.19	1.18	1.27	1.28	1.24	1.20	1.18	0.183	0.049
VFA as a % of total VFAs											
Acetate	49.0	51.2	50.3	50.2	50.1	50.5	49.8	50.3	50.5	1.79	0.369
Propionate	29.5	27.9	28.9	28.6	28.9	28.4	29.0	28.8	28.7	1.23	0.308
Butyrate	14.1	13.4	13.5	13.5	13.6	13.5	13.8	13.5	13.4	0.57	0.140
Iso-butyrate	1.80	1.84	1.75	2.05	1.78	2.03	1.85	1.90	1.88	0.212	0.030
Valerate	2.42	2.41	2.39	2.36	2.39	2.31	2.37	2.34	2.45	0.160	0.792
Iso-valerate	3.21	3.27	3.21	3.30	3.23	3.32	3.24	3.24	3.08	0.134	0.078
Ac : Prop[1]	1.66	1.83	1.75	1.76	1.73	1.78	1.72	1.75	1.77	0.142	0.374
Ac + But : Prop[2]	2.14	2.31	2.22	2.23	2.20	2.26	2.19	2.22	2.24	0.147	0.417

*Gas pressure corrected for initial pressure of 16.41 PSI

[1]Ac : Prop = acetate : propionate ratio, [2]Ac + But : Prop = acetate + butyrate : propionate ratio

[3]PEN = penicillic acid, [4]DON = deoxynivalenol, [5]FUS = fusaric acid, [6]MPA = mycophenolic acid, [7]FMIX = field mix = penicillic acid, deoxynivalenol and fusaric acid, [8]ALT = altogether = penicillic acid, deoxynivalenol, fusaric acid and mycophenolic acid

[9]s.e.d. = standard error of the difference between means, [10]Trt = treatment.

5.7.3. Discussion

5.7.3.1. Methodology in the *in-vitro* fermentation models

Silage for use in the fermenters was dried at 60 °C before being milled through a 1mm screen, to ensure homogeneity of sample and increase the surface area for necessary microbial attachment during the fermentation (Hua et al., 2022). Particularly as the rumen fluid obtained was comprised mostly of liquid phase digesta associated microbial groups; it was important to facilitate attachment to begin fermentation of the silage (McAllister et al., 1994). Grass silage was chosen as a more simplistic feed source in comparison to a TMR, in order to observe any metabolic changes that occurred due to treatment mycotoxin presence, rather than diet. As a TMR contains a higher concentration of rapidly fermentable carbohydrates the effect of mycotoxins on the rumen microbial metabolism could have been masked with a rapid shift toward increased amylolytic microbial activity (Hua et al., 2022) which would have influenced the proportion of fermentation end products (Balch and Rowland, 1957; H. Chen et al., 2021; Ramos et al., 2021). The provision of high starch-content diets are associated with conditions of ruminal acidosis (Jaramillo-López et al., 2017), which have been demonstrated *in-vitro* to compromise the ability of certain microbial groups to metabolise mycotoxins (Debevere et al., 2020a). This was an undesired state for the inoculum to enter in the experiment as the objectives were to evaluate the impact of grass silage mycotoxins on a rumen microbial community composition representative of that of a healthy dairy cow. Furthermore, use of grass silage reduced the risk of an unknown mycotoxin challenge from any contaminated constituents of a TMR (Driehuis et al., 2008).

As the culture was a closed system, any VFAs produced would be unable to diffuse out of the culture (as would occur in the rumen (Storm et al., 2012)), and so a high concentration of short-chain fatty acids would be available more rapidly to the rumen inoculum with a TMR, than if the feed source contained a greater proportion of structural carbohydrates, as with grass silage (Hua et al., 2022). Other methods of *in-vitro* experimentation have been used in which a continuous flow in and out of artificial saliva or feed can be provided (Czerkawski, 2016), which would enable more continuous buffering of the inoculum, however this would have presented difficulties in this experiment in maintaining the mycotoxin concentration.

A reduction from 1 g in the pilot study, to 0.5 g of silage provided in the fermentation vessels also reduced the amount of ‘background’ mycotoxin exposure relative to the inoculum, as a result of the mycotoxins already present in the silage (Table 5-2). It is almost impossible to obtain a farm-produced grass silage without any mycotoxin contamination and autoclaving a silage would undoubtedly alter the physical structure, and nutritional quality. It was therefore

more practical to account for the background mycotoxin exposure than try to sterilise the silage. A number of mycotoxins can also withstand high pressures and temperatures (Kabak, 2009), and so removal of mycotoxins via autoclaving was likely not feasible.

5.7.3.2. Rumen measurements and preparation of the rumen fluid inoculum

Mean rumen fluid pH at collection was within the expected range for both experiment A (mean pH 6.27 ± 0.294) and B (mean pH 6.03 ± 0.304), indicating that cows sampled from in the study were not suffering from any severe rumen dysfunction which could have compromised the activity of the rumen microorganisms in the initial inoculum (Baek et al., 2022; Debevere et al., 2020a).

Cows used in rumen fluid donation were different between experiments A and B which may have influenced the relative abundance of certain microbial populations in the inoculum, due to genetic differences (Li et al., 2019). However, it is likely that the diet the cows had consumed would have been more influential on the microbiota composition present in the inoculum at sampling (H. Chen et al., 2021; L. Wang et al., 2020). Henderson et al. (2015) found that diet had most influence on microbial community composition, even greater than differences between ruminant animal species. For experiment A, the same TMR was provided to all three cows that were donors for the rumen fluid inoculum cultured in the study, and was comprised of 65 % forage, including both grass and maize silages. For experiment B, again the three donor cows were fed the same TMR, which differed from the TMR in part B as comprised of 63 % forage. The ratio of forage to concentrate provided to donor cows was therefore similar between experiment A and B, suggesting that there would not have been large differences in the relative proportions of the core rumen phyla such as Bacteroidetes and Firmicutes, entering the inoculum in either experiment A or B, that have been shown to be affected by forage to concentrate ratio (L. Wang et al., 2020).

As the rumen inoculum from the three donor cows had not undergone any adaptation phase to the grass silage provided in the IFM fermentation, there may have been a selection pressure imposed on the inoculum. However, the presence of a variety of forages (including grass silage) and concentrates in the TMR fed to cows in experiments A and B, would likely result in a diverse starting inoculum, and able to adapt to the grass silage provided in the fermenters. This is supported by L. Wang et al. (2020) whom demonstrated a higher microbial diversity in dairy cows fed a high forage diet (57 % maize silage) as opposed to a high concentrate diet (19 % maize silage). The combination of rumen fluid of the three donor cows into one inoculum, was also thought to also increase the species richness of the initial inoculum, especially where the three cows in experiments A and B varied in their stages of lactation

(Bainbridge et al., 2016). A non-selective buffer with the inclusion of clarified rumen fluid (Hungate, 1960), was chosen to provide microbial growth-promoting factors that remain currently unidentified but have been demonstrated to increase species richness, albeit when plating microbial cultures (Zehavi et al., 2018). Rumen fluid for the inoculum was collected approximately 3 hours post feeding as the abundance of culturable rumen bacteria was likely to be highest at this time point, as was demonstrated by Bryant and Robinson, (1961) and more recently by Belanche et al. (2019). Any *in-vitro* experiments will inherently apply a selection pressure to an extent on the microbial population introduced, as not all rumen microbes can be successfully cultured outside of the rumen environment (Hackmann et al., 2024; Zehavi et al., 2018) and so the nature of the study made this unavoidable.

An advantage of *in-vitro* study is that it allows for the addition of mycotoxin concentrations that may be detrimental to the health of an animal, had the same concentration been administered *in-vivo*. In experiment A, for example concentrations of DON at the extreme level of inclusion (10 µg/g DM) were twice the maximum guidance value recommended by the EU for DON contamination in compound feeds for livestock (5 mg/kg at 88 % DM; European Union (EU) (2006). Due to the scarcity of information regarding the effect of grass silage mycotoxins on the rumen metabolism, it was decided a series of *in-vitro* studies would be sufficient to provide a foundation for understanding these effects.

5.7.3.3. Selection of mycotoxins and determination of level of inclusion

Though other *in-vitro* experiments in the literature have calculated the level of inclusion relative to the total culture volume, they have focussed on the ability of the rumen microbiome to degrade the supplied mycotoxin (i.e. a reduction in mycotoxin concentration; (Caloni et al., 2000; Hult et al., 1976; Kiessling et al., 1984)). As this experiment focussed on the impact of mycotoxins on the rumen microbial metabolism, the concentrations were calculated with respect to the DM of the feed provided, providing clearer interpretation of contamination levels relative to the values provided in commercial mycotoxin testing (i.e. Alltech© 37^{+} results, µg/kg DM), and the EU guidance levels (i.e. European Union (EU) (2006), mg/kg DM). As explained previously, the medium and high levels of inclusion were determined from results of the farm survey in Chapter 3 and with input from the literature, as shown in Appendix Table 4. However, for the extreme levels of inclusion, values were challenging to determine for FUS, PEN and MPA due to the lack of EU legislation or recommendation advising thresholds for these three mycotoxins in ruminant diets. In absence of this, extreme levels of inclusion for FUS, PEN and MPA were devised by maintaining the same magnitude of increase to the extreme level of inclusion, as had been calculated for DON. This could mean that the true values that would

be considered “extreme” for PEN, FUS, and MPA exposure in dairy cows, are substantially higher in practice, but this remains to be elucidated in future research.

Penicillic acid present already in the grass silage provided at a concentration of 2,006 µg/kg DM (2.01 µg/g DM; Table 5-2) meant that the total dose of PEN in the fermenters, could have ranged from 1.00 – 3.01 µg/g at the medium level of inclusion, 4.00 – 6.01 µg/g at the high level of inclusion, and 16.00 – 18.01 µg/g at the extreme level of inclusion in the fermentation cultures. Nevertheless, the theoretical ranges at each level are still within the order of magnitude for each level of inclusion and so are unlikely to influence the contamination too greatly to have required a reclassification of the level of inclusion for each of the medium, high or extreme levels. All other mycotoxins present in the silage provided (ergocryptin(in)e, fumonisin B_1, fumonisin B_2 and FUS; Table 5-2) were between 0.002 and 0.134 µg/g DM and so can be considered negligible when comparing the main effects of the addition of the mycotoxin treatments.

There may have been an interaction effect between the PEN present in the silage and the experimental treatment addition of MPA, FUS or DON (Speijers and Speijers, 2004), but it was extremely challenging to obtain silage from the survey farms that contained a lesser concentration of PEN, without higher concentrations of other grass silage mycotoxins – PEN was present in 71.6 % of silages surveyed (Chapter 3). PEN has been demonstrated to have synergistic effects with ochratoxin A in mice, leading to fatality (Sansing et al., 1976). However, this was not applicable to this study, and information on synergistic effects of PEN with the other mycotoxins present in the silage is lacking in the literature.

The combinations of mycotoxins in part B that were chosen were determined by common co-occurrences of the mycotoxins identified in grass silages from Chapter 3. The mycotoxins FUS and PEN were found in over 70 % of the total clamps survey and were found to co-occur most commonly, where both were present in 50 % of the total clamps surveyed, as well as DON being present with both PEN (in 16 % of clamps surveyed) and FUS (in 14 % of clamps surveyed). The “Field Mix” treatment was designed to most likely replicate the combinations of prominent mycotoxins that the rumen microbiota would be exposed to if a cow had consumed grass silage in Great Britain. The “altogether mix” included MPA, as despite only being found in 2.70 % of total clamps surveyed, it has been found to contaminate grass silage previously (Schneweis et al., 2000) and the immunosuppressive effects of MPA are well detailed (Bentley, 2000), however, information on its impact on rumen fermentation is lacking. To the authors knowledge, the only study to date was by Mohr et al. (2007) whom investigated haematological parameters and body weight in sheep after exposure to MPA, and not

fermentation impacts. Mycophenolic acid was also the main mycotoxin treatment provided in Chapter 6 during an *in-vivo* trial. (Chronologically, the experiment in Chapter 6 was carried out before the *in-vitro* investigations in this Chapter). The high levels of inclusion were used for all treatments in experiment B to ensure applicability of findings to realistic levels on-farm, as in Chapter 3.

5.7.3.4. Gas production

Equations for the calculation of gas volume produced from gas pressure, are a topic of great debate in the literature, as well as the use of either vented culture systems or closed systems for the most "accurate" representation of the *in-vivo* microbial activity in an *in-vitro* model. Romero-Pérez and Beauchemin, (2018) calculated two additional equations with the aim of standardising the calculation of gas volume produced across *in-vitro* experiments from gas pressure, however, the equations can only be relied upon when the same experimental conditions as the author carried out, are implemented. It is generally accepted that Boyle's Law (Equation 13) can be used to calculate the volume of gas produced, when the starting pressure, end pressure and starting volume is known, but this ignores any gas that may have dissolved back into the culture and so could underestimate total production (Romero-Pérez and Beauchemin, 2018). Therefore, the total gas pressure (PSI) after a 48 h incubation was presented for this study, to prevent any underestimation of total microbial activity.

Equation 13. Boyle's Law for calculating the volume of gas produced from gas pressure at a constant temperature (Webster, 1963).

$$P_1 V_1 = P_2 V_2$$

Where P_1 *= start point pressure,*
P_2 *= end point pressure,*
V_1 *= start point volume,*
V_2 *= end point volume*

Though an accumulation of gas pressure in the headspace has been suggested to impact on rumen microbial activity (Theodorou et al., 1994), the IFM procedure does not allow for venting. Furthermore, closed systems have been shown to provide better measurements of methane production in the total headspace (Cattani et al., 2014) which were used to infer methanogenic activity and potential protozoal activity within the cultures that would impact on overall rumen fermentation.

If using Boyle's Law to calculate volume of gas produced and expressed on the DM of the substrate inoculated, then the total gas production ranged between 60.4 ml to 64.6 ml/ g DM, in experiment A, and between 60.0 ml to 70.0 ml/g DM in experiment B. These values are lower than that reported in a meta-analysis by Maccarana et al. (2016) whom standardised studies as "ml of gas produced per g of substrate DM". However, the lack of venting the gas throughout the 48 h fermentation is known to lead to underestimations in total gas produced due to dissolution of CO_2 back into the inoculum, whilst substrate type and concentration of inoculum volume also have a large influence on gas production (Cone et al., 1996), making comparisons between experiments challenging. Moreover, as PEN possesses antibacterial properties (Cole et al., 2003) the background inclusion of PEN in the substrate may have suppressed overall microbial activity. Nonetheless, there was no difference observed between the gas production of the control and the mycotoxin treated cultures in experiment A or B, with ranges between 8.06 to 9.67 PSI and 6.19 to 8.97 PSI, respectively. This study therefore suggests no impact of mycotoxin addition on rumen gas production activity.

5.7.3.5. Methane concentration of the headspace gas

No difference in methane production as a result of mycotoxin treatment in either experiment A or B could be demonstrated which would suggest a lack of effect of these mycotoxins on rumen methanogens. However, owing to the difficulty of culturing protozoa and associated methanogenic archaea outside of the rumen (Hackmann et al., 2024) it is more likely that there was a decline in viability of these organisms in the cultures over the 48 h incubation. Moreover, the most predominant methanogen genus in the rumen is *Methanobrevibacter* which is rarely found exclusive of a symbiosis with rumen protozoa and fungi (Henderson et al., 2015; Ungerfeld, 2018).

Had there been a healthy methanogenic population, a relative increase in methane in comparison to CO_2 in the headspace of the cultures may have been observed, as CO_2 is utilised in the production of CH_4 (Morgavi et al., 2010). In this case, concentrations of methane would be expected to exceed 20 – 30 % of the rumen fermentation gas concentration (Min et al., 2022) as no expulsion of gases were able to occur, unlike *in-vivo*, where gases are eructed by the ruminant (D'Souza et al., 2022). With a CO_2 infused headspace, and a substrate of alfalfa hay only, Patra and Yu (2013) demonstrated a methane headspace concentration of 23.9 %. In contrast both in study A and B across all treatments, methane concentrations did not exceed a maximum of 6.61 % of the total headspace gas, supporting that there was a reduction in methanogenic activity in the fermentation cultures. If methanogen activity had

ceased due to the experimentation model, then no effect of mycotoxin addition on the production of methane can be concluded from this study.

There is a possibility that methanogens could have been affected by the background mycotoxin contamination in the substrate of predominantly PEN, that is known to possess antibiotic properties. A study by Rusanowska et al. (2020) demonstrated a reduction in methanogenic activity with three antibiotics but this consequently resulted in an accumulation of differing VFAs dependent on antibiotic used. In contrast, in study B there was a trend for a lower total VFA content in comparison to the control, and in some treatments involving PEN, there was a significant decrease in propionate, butyrate and isovalerate in comparison to the control. This again supports that there was an overall decrease in viable methanogenic organisms in the cultures, likely as a result of being cultured outside of the rumen environment. A future study involving molecular techniques to establish methanogen, and protozoal abundance should be considered.

5.7.3.6. Culture VFA concentrations

In experiment B there was a trend for lower total VFAs in cultures treated with mycotoxins in combination, compared to the control in PEN x DON and PEN x MPA treatments; also supported by a significant reduction in propionate and isovalerate concentrations of these treated cultures. Cultures treated with PEN x FUS, PEN x MPA, PEN x DON and the FMIX demonstrated a lower butyrate concentration and there was a trend for a reduction in valerate concentration with PEN x DON and PEN x MPA treatments. Other in-vitro studies have found mixed results with the effect of mycotoxins on VFAs. For example, Boguhn et al. (2010) demonstrated a reduction in concentration of isobutyrate, without effect on any other VFA with DON between 5.8 and 6.9 mg/kg DM in diets at two concentrate levels, in a sheep *in-vitro* model. Hartinger et al. (2022) demonstrated a lower total VFA concentration in Holstein dairy cows fed a TMR contaminated with ZEA and FUM. In an *in-vitro* study using Holstein rumen fluid, Jeong et al. (2010) demonstrated a reduction in both acetate and propionate concentrations with DON addition at 40 mg/kg DM. Differences in diets provided, concentrations and type of mycotoxin administered, and a lack of studies on grass silage associated mycotoxins, mean there is difficulty in making comparisons of previous studies with this study. It is hoped that this study forms a basis for further research into the effect of grass silage mycotoxins on rumen fermentation. In any case, it is clear that well-studied mycotoxins (such as those commonly found in grains and maize) have been demonstrated to impact on the VFA production in the rumen in *in-vitro* and *in-vivo* (Hartinger et al., 2023; Rivera-Chacon

et al., 2024) settings, and this study reveals that this is also true for grass silage mycotoxins when in combination with one another.

As ruminal VFA production contributes approximately 60-70 % of the metabolisable energy for the dairy cows (Van Soest, 1994), a lower overall VFA concentration may result in reduced performance including a reduced milk yield (Seymour et al., 2005) and a reduction in butyrate concentration may also lead to a reduction in milk fat content (Miettinen and Huhtanen, 1996). Additionally, a reduction in propionate concentration available could affect glucose levels in the cow, as propionate production is correlated with gluconeogenesis (Zhang et al., 2015). Additionally, it may lead to increased rumen methane production as more H_2 is available for hydrogenotrophic methanogenesis (Morgavi et al., 2010). Combined with known immunosuppressive activity of MPA (Bentley, 2000), and genotoxic effects of DON (Kiessling, 1986), these effects on performance may be exacerbated when administered to a cow *in-vivo.* Further research is required to elucidate the mechanisms of interaction between the rumen microorganisms and the combinations of grass silage mycotoxins, but this is the first study to demonstrate that combinations of common grass silage mycotoxins impact on rumen fermentation, compared with when administered alone, even when administered at higher levels.

Interestingly, there were no differences in ALT combinations in comparison to the control for any of the fermentation parameters investigated, suggesting more complex synergistic or antagonistic interactions between the mycotoxins when all four are included (Speijers and Speijers, 2004). This may have led to the negation of some mycotoxin effects on the rumen fermentation seen in the dual combinations, and again provides an avenue for further investigation when assessing the risk of a silage on dairy cow performance.

5.7.3.7. Culture VFA proportions

There was however no effect of mycotoxin treatment in experiment A or B on the proportion of VFAs produced, indicating that there was not a large shift in overall fermentation activity that could lead to dysbiosis of the rumen. No alteration in the relative abundance of Firmicutes spp. to Bacteroidetes spp. could be inferred (Den Besten et al., 2013; Magne et al., 2020) as the activity of both propionate and butyrate producing bacteria were seemingly negatively impacted by mycotoxin addition. This does not mean that certain groups within these phyla were not necessarily more sensitive to the addition of certain mycotoxin treatments, however. For example, penicillin was demonstrated as an ineffective antibiotic against species of *Bacteroides,* and *Prevotella,* but was demonstrated effective in inhibiting growth of *Propionibacterium* spp by Roberts et al. (2006). It would be tempting to suggest that PEN may

have acted similarly in this study, resulting in the lowered concentration of propionate through the inhibition of *Propionibacterium* species, but bacteria demonstrate differences in sensitivity between penicillin and PEN (Ezzat et al., 2007), as well as these compounds differing in chemical structure. Commenting on the effect of the mycotoxin treatments on community structure based on fermentation end-products alone, can therefore be misleading. Community analysis was beyond the scope of this study but would be an excellent avenue for further investigation.

Overall, production of the three most abundant VFAs in the rumen (acetate, propionate and butyrate (Van Soest, 1994)) were all reduced in terms of expected ranges for that of rumen fluid, even considering the dilution factor of 80% rumen fluid to 20 % buffer of the inoculum. For example, acetate production across experiments A and B in an 80 % rumen fluid inoculum ranged from 18. 3 to 26.7 mM, which would have expected to have been 48 to 72 mM at 80 %, considering reference values quote pure rumen fluid acetate concentrations between 60 and 90 mM (McSweeney and Mackie, 2012). Furthermore, the substrate having been grass silage and thus higher in fibrous content would have meant that acetate concentrations should have been toward the higher end of the range (Moran, 2005; Sutton et al., 2003). The concentration of bicarbonate in the culture buffer has been shown to impact on the production of VFAs *in-vitro* (Patra and Yu, 2013) and may provide a reason as to why levels of VFA expected were not seen. In contrast however, concentrations of acetate, propionate and butyrate in this study mostly align with the ranges presented by Jeong et al. (2010) in an *in-vitro* study on DON addition, where the substrate was pure cellulose (propionate concentrations were slightly higher, in this study in comparison). Nevertheless, a reduction in propionate, butyrate and isovalerate concentrations, and a trend for an overall decrease in VFA production was still identified in this study when cultures were treated with PEN x MPA and PEN x DON mixtures, and in the case of butyrate, also PEN x FUS.

5.7.3.8. Conclusions and opportunites for future studies

To the authors knowledge, this study is the first to investigate the effects of the four common grass silage mycotoxins of FUS, PEN, MPA and DON on rumen fermentation *in-vitro*. When mycotoxins were added in isolation, no effects on gas production, pH, methane concentration, ammonia production or VFAs were demonstrated, from a level of inclusion common in grass silage to a level of contamination not expected to occur naturally. However, when in combination with one another, as is found in grass silage naturally, combinations of PEN x DON, PEN x MPA, PEN x FUS and FMIX resulted in various suppression of the production of propionate, butyrate, and isovalerate, with additional trends for a reduction in valerate and

total VFAs in the rumen. This study highlights the importance of including mycotoxin treatments in combination for any appropriate assessment of the risk posed to dairy cows. Finally, grass silages in Great Britain contaminated with commonly found mycotoxins at naturally occurring concentrations pose a risk to the rumen fermentative activity and subsequent performance of dairy cows.

Chapter 6

The effects of a short-term feeding phase of grass silage high or low in mycotoxins, with or without additional mycophenolic acid or a mycotoxin binder, on the rumen microbial metabolism and performance of dairy cows.

6. The effects of a short-term feeding phase of grass silage high or low in mycotoxins, with or without additional mycophenolic acid or a mycotoxin binder, on the rumen microbial metabolism and performance of dairy cows

6.1. Introduction

Following on from Chapter 5, where combinations of mycotoxins were found to impact on the rumen fermentation *in-vitro*, it was important to understand if these effects were also demonstrated *in-vivo*. The controlled nature of *in-vitro* studies mean that mycotoxins are often added exogenously (Debevere et al., 2020b). Disadvantageously, this often results in the exclusion of accompanying fermentation characteristics of a silage or diet representative of on-farm contaminated silage (Gallo and Masoero, 2010; Riccio et al., 2014).

As identified in the Chapter 1, there are few *in-vivo* studies that have investigated the effects of mycotoxins, on the rumen metabolism and microbiome *in-vivo*. Hartinger and colleagues have recently published studies using molecular techniques to identify the changes in dairy cow rumen microbial composition upon exposure to mycotoxins *in-vivo*, but to date only ZEA and FUM have been investigated (Hartinger et al., 2023, 2022). This year, Dong et al. (2024) investigated DON exposure on rumen function using metabolomics and molecular analyses, but this was *in-vitro.* May et al. (2000) did investigate the effect of FUS, DON and picolinic acid (from which FUS is a derivative) on two prominent rumen microorganisms *Ruminococcus albus* (phylum: Firmicutes) and *Methanobrevibacter ruminantium* (methanogenic archaeal species), but again, these studies were carried out *in-vitro* and exclusive of the full rumen ecological network. Additionally, the impact of *Penicillium* or *Aspergillus* associated mycotoxins, present in grass silages, have not yet been investigated on the rumen microbial composition *in-vivo*. As such, there is a dearth of information on the effect of mycotoxins on the rumen microbial composition, let alone information on the effects of common grass silage mycotoxins.

To mitigate the negative effects of mycotoxin exposure, a variety of binders and enzymatic agents are commercially available and have been comprehensively reviewed by De Mil et al., (2015) and Whitlow, (2006). One such binder investigated by Jouany et al., (2004) involves the beta-D-glucan structure of cell walls of the yeast *Saccharomyces cerevisiae,* which were found to form chemical bonds with certain mycotoxins such as ZEN, limiting interaction of the mycotoxin with the rumen microbiome (Yiannikouris et al., 2005). The efficacy of yeast cell-wall binders (YCW binders) has been evaluated both *in-vitro* and *in-vivo* with an *in-vivo* investigation into the ability of a YCW binder to reduce secretions of aflatoxin M_1 into the milk of Ayrshire dairy cows, which resulted in significant reduction when the binder was fed in a

diet dosed with AFB_1, without affecting cow performance (Moran et al., 2013). More recently, Debevere et al (2020b), evaluated the *in-vitro* efficacy of different mycotoxin mitigation products. A clay and YCW binder was found to have reduced the concentration of enniatins and roquefortine C in rumen fluid, but not that of mycophenolic acid (MPA), DON or ZEN. As MPA is a prominent grass silage mycotoxin, it is important to assess the risk grass silages pose to the rumen fermentation when a YCW binder is incorporated *in-vivo*.

An *in-vivo* study was therefore required to assess the specific risk that naturally contaminated grass silage poses to dairy cow performance with a focus on the impact on the rumen microbiome and metabolism.

6.2. Materials and methods

6.2.1. Hypotheses and objectives

The hypotheses for this study were that:

1) Grass silage contaminated with mycotoxins would result in altered rumen fermentation due to a disruption to the rumen microbial composition
2) Grass silage contaminated with mycotoxins would impact on dairy cow performance
3) The addition of a mycotoxin binder would reverse any effects on the rumen fermentation, and animal performance that had been induced by mycotoxin exposure.

The objective of the study was to assess the effect of a short-term feeding period (5 d) of mycotoxin contaminated grass silage on the rumen microbiome and metabolism in five lactating, rumen fistulated, Holstein-Friesian dairy cows as part of a Latin square design. Additionally, a grass silage mycotoxin (MPA) was added at a rate of 5000 µg/kg of DM TMR to increase the mycotoxin load of the diet, and a commercially available mycotoxin binder (Mycosorb A^+ (2021 formulation), Alltech© UK Ltd.) was included to evaluate the mitigation of any mycotoxin-induced effects on dairy cow performance.

6.2.2. Forages

At Harper Adams University Farm (Shropshire, United Kingdom), a second cut grass sward comprised mostly of *Lolium perenne* spp. was mown, wilted for approximately 30 h and precision chopped. The grass was ensiled in one of two roofed, concrete walled clamps of 10 m x 5 m x 2 m on the 24th of June 2020. One clamp (Control) was filled with ~30 t fresh weight (~10.6 t DM) of grass, rolled well, sealed with an oxygen barrier film and plastic sheet, and weighed down with a layer of large square bales within 2 h of ensiling. The second (Spoiled)

clamp was filled with ~8 t fresh weight (~3 t DM) grass, received minimal compaction and had a previously mycotoxin contaminated grass silage (Appendix Table 5) spread evenly across the layers at a rate of ~1/kg fresh weight, to “spike” the silage clamp. The spoiled clamp was left unsheeted for 24 h post-ensiling, and received no additional surface weight.

6.3. Animals and study design

All procedures concerning the use of animals for this research, were conducted in accordance with the Animals (Scientific Procedures) Act of 1986 (amended 2012) of the United Kingdom (available at: www.legislation.gov.uk/ukpga/1986). All procedures were also approved by the Harper Adams University local ethics committee.

Five multiparous Holstein-Friesian dairy cows that had previously been fitted with permanent 10 cm diameter rumen cannula (Bar Diamond, USA) and were 255 ± 26.7 d post-calving were used in a Latin square design with 5 periods each of 23 days duration (Table 6-3). During the first 18 d of each period all cows were fed the Control grass silage as part of a TMR (Table 6-1) formulated for a 675 kg dairy cow yielding 27 kg/d of milk, according to Thomas, (2004). During the final 5 d of each period (challenge phase) the cows received one of 5 dietary treatments as outlined in Table 6-2.

Table 6-1. Ingredient composition of the total mixed ration (DM basis) of the Control and Spoiled treatments fed to dairy cows over a five-day mycotoxin challenge phase on the rumen metabolism and microbiome and subsequent effect on dairy cow performance.

TMR[1] constituents (g/kg)	Treatment TMR	
	C[2]	S[3]
Control grass silage	640	-
Spoiled grass silage	-	640
Rolled barley	166	166
Molassed sugar beet pulp	71.0	71.0
Faba fibre[4]	119	119
Minerals/vitamins	4.00	4.00

[1]TMR = Total mixed ration
[2]C = Control silage dietary treatment;
[3]S = Spoiled silage dietary treatment;
[4]Vicia Faba pellets from KW Alternative Feeds, Leeds, United Kingdom

Table 6-2. Treatment outline for assessing the impact of a five-day mycotoxin challenge phase on the rumen metabolism and microbiome and subsequent effect on dairy cow performance.

Treatment code	Treatment details
C	Control silage
CM	Control silage + MPA[1] 5000 µg/kg DM[2] TMR[3]
S	Spoiled silage
SM	Spoiled silage + MPA at 5000 µg/kg DM TMR
SMB	Spoiled silage + MPA at 5000 µg/kg DM TMR + 150 g/head/d MB[4]

[1]MPA = mycophenolic acid (Cat: 459380250, Acros Organics, Thermo Fisher Scientific, Massachusetts, United States);
[2]DM = dry matter;
[3]TMR = total mixed ration;
[4]MB = mycotoxin binder (Mycosorb A+ (2021 Formulation) Alltech© UK Ltd., Lincolnshire, United Kingdom).

Table 6-3. Latin square design with dietary treatments for assessing the impact of a five-day mycotoxin challenge phase on the rumen metabolism and microbiome and subsequent effect on dairy cow performance.

Cow	Challenge period				
	1	2	3	4	5
Cow A	C[1]	SMB	CM	S	SM
Cow B	S[2]	C	SMB	SM	CM
Cow C	SMB[5]	SM	C	CM	S
Cow D	SM[4]	CM	S	SMB	C
Cow E	CM[3]	S	SM	C	SMB

[1]C = Control silage, [2]S = Spoiled silage, [3]CM = Control silage + mycophenolic acid at 5000 µg/kg DM TMR, [4]SM = Spoiled silage + mycophenolic acid at 5000 µg/kg DM TMR, [5]SMB = Spoiled silage + mycophenolic acid at 5000 µg/kg DM TMR + 150 g/head/d mycotoxin binder.

The ratio of concentrates to forage was maintained at 36:64 for all treatments during the challenge phase (Table 6-1).

For the first 18 d of each period, cows were group housed in a sawdust bedded loose yard with a concrete standing area in front of the feed face, and feed accessible via individual feed gates (American Calan, New Hampshire, USA). During the challenge phase the cows were restrained and fed in individual stalls fitted with rubber mattresses, bedded with sawdust, and with unlimited access to fresh water.

6.3.1. Experimental routine

The TMR was mixed and fed daily at approximately 0900 h using a HiSpec® feed mixer wagon (HiSpec® Engineering Ltd, Co. Carlow, Ireland) at 105% of the previous day's recorded intake (Figure 6-1). During the challenge phase TMR was spread evenly onto plastic feed sheets daily, prior to adding MPA (Sigma-Aldrich®, Dorset, United Kingdom) to treatments CM, SM and SMB at a rate of 5000 µg/kg DM in 250 ml aqueous suspension; mixed thoroughly to ensure even coverage. For treatments C and S, 250 ml of distilled water was added *in lieu* and mixed thoroughly. A mycotoxin binder (Mycosorb A^{+}; Alltech© Ltd., Lincolnshire, United Kingdom) was added to the SMB diet at a rate of 150 g/head/day as per the manufacturer's recommendation and mixed thoroughly prior to feeding.

The cows were milked twice daily using a portable milking unit (Milkline, London, United Kingdom) at 0600 and 1600 h, with yield recorded at each milking. Milk samples were collected at each milking on day 17 of each period (1 d before the challenge phase), days 1, 3 and 5 of the challenge phase, and 3 d after the challenge phase for subsequent analysis of milk composition (Figure 6-1). Fat, protein, casein, lactose, somatic cell count (SCC) and urea were determined in milk samples via near mid-infra-red analysis at the National Milk Laboratories (NML, Staffordshire, United Kingdom).

Cow body temperature was recorded daily during the challenge phase at both milkings using a thermometer inserted ~5 cm into the rectum. Cow body weight was recorded using a Tru-Test© electronic cattle weigh-plate (Datamars Agri, Scotland, United Kingdom) 1 d prior to, and on the final day of the challenge phase. Body condition was scored by the same technician 1 d prior to and on the final day of the challenge phase, as described by Ferguson et al., (1994).

Rumen fluid samples were collected twice on days 1, 2 and 5 of the challenge phase, and 3 d after the challenge phase, at approximately 3 and 7 h post feeding (Figure 6-1). Two grab

samples were obtained via rumen fistula, at each time point from just underneath the surface mat of solid digesta in the centre (dorsal) and at the bottom of the solid mass (approx. 52 cm deep) into the ventral region of the rumen and strained through 4 layers of muslin into 10 L plastic buckets.

Rumen fluid pH was recorded immediately post sampling with a pH meter (Jenway 3510, Antylia Scientific, Illinois, United States). Strained rumen fluid (45 ml) was stored with 5 ml of 25% w/v metaphosphoric acid (Sigma-Aldrich®, Dorset, United Kingdom) for subsequent VFA and ammonia-N analysis (intra-assay % CV: 1.02) as described in Chapter 2. Thirty-five millilitres of strained rumen fluid was poured into 50 ml tubes and topped up with 15 ml 30% w/v glycerol (Sigma-Aldrich®, Dorset, United Kingdom) suspension for subsequent DNA extraction. All samples were frozen at -20°C immediately after collection.

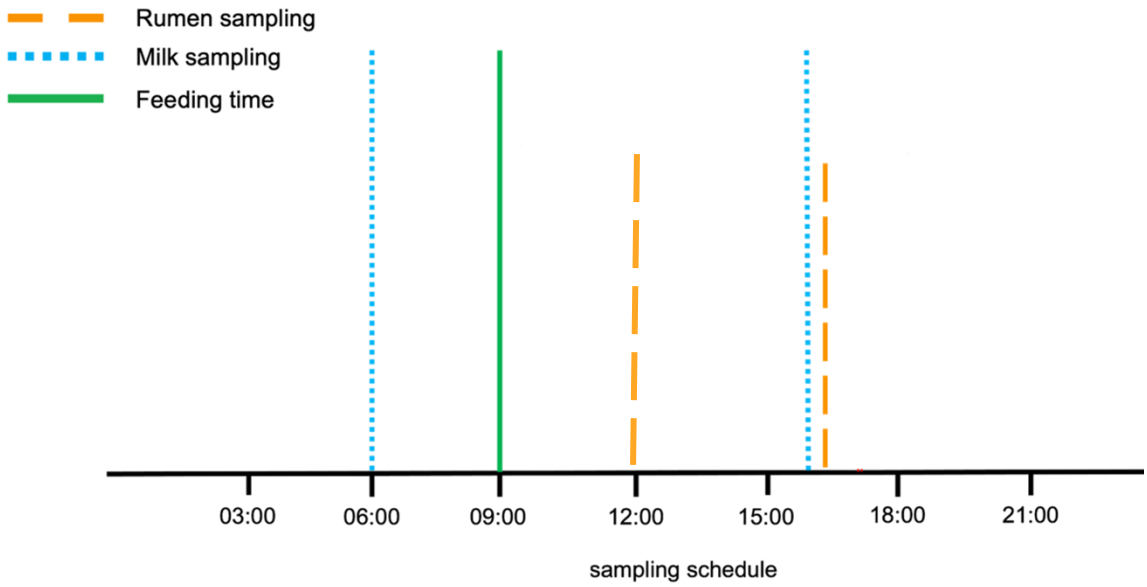


Figure 6-1. The sampling schedule for assessing the impact of a five-day mycotoxin challenge phase on the rumen metabolism and microbiome and subsequent effect on dairy cow performance. (Note: Rumen sampling was only carried out on d 1, 2, 5 and 3 d after the challenge phase).

6.3.2. Chemical analyses

Control and spoiled silage were bulked across the 5 days of each challenge period and analysed for DM, crude protein (intra-assay % CV: 2.75) and ash according to the Association of Official Analytical Chemists, (AOAC, (2012), methods: 934.01, 988.05, 924.05, respectively) and neutral detergent fibre determined according to Van Soest et al., (1991) using heat-stable α-amylase (Sigma, Gillingham, United Kingdom) and sodium sulfite, and expressed exclusive of residual ash, as described in Chapter 2 (intra-assay % CV: 2.13). Bulked samples of each two silages were also analysed for their mycotoxin concentration by Alltech© 37+, Dublin, Ireland by LC MS-MS as described in Chapter 2. Samples were also sent to Sciantec, (Cawood Laboratories, North Yorkshire, United Kingdom) for analysis of lactic acid, VFAs, and alcohols.

6.3.3. Molecular analyses

Microbial DNA was extracted using a repeated bead beating and column purification method based on Yu and Morrison, (2004) and Qiagen's QIAamp Fast DNA Stool Mini Kit (Qiagen®, Mettman, Germany) following the manufacturers' protocol. The extracted DNA was amplified in triplicate 25 µl polymerase chain reactions (T100™ Thermo Cycler, Bio-Rad®, California, United States) using high-fidelity DNA polymerase (Q5®, New England BioLabs, Massachusetts, United States) and dual indexed universal prokaryotic primers targeting the V4 variable region of the 16S rRNA gene (Kozich et al., 2013). Primer sequences are detailed in Appendix Table 6. Quantification of amplicons was carried out using Quant-IT™, PicoGreen™ (Thermo Fisher Scientific, Massachusetts, United States), and were normalised and pooled in equimolar concentrations ready for sequencing.

Quality assessment of the pooled amplicons was carried out using an Agilent 2100 Bioanalyser System (Agilent Technologies™, California, United States) and sequenced using the Illumina MiSeq® V2 250 paired-end reagent kit (Illumina Inc®, United States) at Edinburgh Genomics (University of Edinburgh, Scotland, United Kingdom).

Raw sequence data were processed using *mothur* (Schloss et al., 2009) for quality control and filtering, chimera removal and clustering into operational taxonomic units (OTUs) at 97% species identity. Taxonomic classification of the representative sequences was carried out using the SILVA 132 SEED reference database (Henderson et al., 2019; Yilmaz et al., 2014). Taxonomic classification of selected OTUs was undertaken using BLASTn against type material from the standard nr database (National Centre for Biotechnology Information,

Maryland, United States). The sequence data are openly available at www.ebi.ac.uk/ena/browser/view/PRJEB59825.

6.3.4. Statistical analyses

All data were analysed using GenStat (21st Edition, VSN International, Hertfordshire, United Kingdom) using analysis of variance. Where data spanned multiple days along the challenge phase, analysis involved repeated measures as in the model below:

$$Y_{ijkm} = \mu + T_i + C_j + P_k + D_m + (TD)_{im} + \varepsilon_{ijkm}$$

Measurements recorded on day 17 of each period were used as a co-variate where appropriate as in the model below:

$$Y_{ijkm} = \mu + T_i + C_j + P_k + D_m + (TD)_{im} + covariate_{ijkm} + \varepsilon_{ijkm}$$

Where, Y_{ijkm}= dependent variable; μ = overall mean; T_i = main effect of the i-th dietary treatment (C, CM, S, SM, or SMB), C_j = random effect of the j-th cow (cows A to E); P_k = random effect of the k-th period (periods 1 to 5); D_m = main effect of the m-th day (repeated measures across the challenge period); $(TD)_{im}$ = interaction effect between dietary treatment (T) and days along the challenge phase (D); $covariate_{ijkm}$ = corrected for measurements recorded on day 17 of each period; and ε_{ijkm}= residual error.

Significance was considered at $P < 0.05$ and a trend considered at $P < 0.10$ but > 0.05. Tukey's test was conducted *post-hoc* to identify treatment means that differed significantly.

Differences in rumen microbial alpha diversity was measured using OBS, Chao1, Shannon and inverse Simpson metrics and analysed using analysis of variance. Linear discriminant analysis effect size (LEfSe, Segata et al., 2011) was used to identify taxonomic biomarkers based on differences in the relative abundance of OTUs ($P < 0.05$) between treatments grouped by either an effect of MPA addition (C vs CM and S vs SM), an effect of silage type (C vs S and CM vs SM) or an effect of mycotoxin binder addition (SM vs SMB). Values were considered significant at a Linear Discriminant Analysis (LDA) score ≥ 2.0 or ≤ -2.0.

6.4. Results

6.4.1. Forage analysis and mycotoxin concentration

The control silage mean DM content was 302 g/kg, 62 g/kg lower ($P = 0.016$) than the spoiled silage at 364 g/kg and also 16 g/kg lower in crude protein content ($P = 0.005$) with means of

203 g/kg DM and 219 g/kg DM for control and spoiled silage, respectively. Neutral detergent fibre content was higher in control silage with a mean of 393 g/kg DM in comparison to 367 g/kg DM in the spoiled silage. Ash content was not found to be different between the silages ($P = 0.942$) with means of 13.8 g/kg DM and 14.0 g/kg DM for the control and spoiled silages respectively. Likewise, there were no differences found between lactic acid concentration of the control and spoiled silages ($P = 0.094$), with means of 60.6 g/kg DM and 67.0 g/kg DM respectively. Acetic acid content was higher ($P < 0.001$) in control silages with a mean of 37.1 g/kg DM in control grass silage compared with 20.8 g/kg DM in the spoiled and similarly propionic acid content was higher ($P = 0.036$) in control silage with a mean of 1.63 g/kg DM and a mean of 0.910 g/kg DM in spoiled. There were no differences found between concentrations of butyric acid ($P = 0.340$) in the two silages with 0.079 g/kg DM in the control and 0.103 g/kg DM in the spoiled silage. Isobutyric acid was below the level of detection in both control and spoiled silages and there were no differences found for valeric acid ($P = 0.340$) and isovaleric acid ($P = 0.182$) between the two silages.

Ethanol was higher in the control silage ($P = 0.016$) with a mean of 2.97 g/kg DM in comparison to a mean of 1.76 g/kg DM in the spoiled. Similarly, propan-1-ol concentrations were higher in control silage ($P = 0.033$) with a mean of 1.34 g/kg DM, in comparison to a mean of 0.370 for the spoiled. Propane-1,2-diol concentrations were higher in control silage ($P < 0.001$) with a mean of 19.4 g/kg DM in comparison to 2.70 g/kg DM in the spoiled silage. The pH of silage was higher in the spoiled silage ($P = 0.043$) with a mean pH value of 3.97 in comparison to 3.84 for the control silage.

More mycotoxins were identified in the spoiled silage, with a higher concentration of DON ($P = 0.018$), FUS ($P < 0.001$), AFB_1, MPA and roquefortine C, with the latter three absent from the control silage. There was a trend for a lower concentration of moniliformin in the spoiled silage ($P = 0.077$) in comparison to the control. There was no difference in the concentration of enniatin A/A_1 ($P = 0.675$) or enniatin B/B_1 ($P = 0.637$) between the two silages. There was a trend ($P = 0.076$) for the REQ of the spoiled silage to be higher than the control.

Table 6-4. Chemical composition and concentration of volatiles and mycotoxins in the Control and Spoiled grass silages used in dietary treatments for assessing the impact of a five-day mycotoxin challenge phase on the rumen metabolism and microbiome and subsequent effect on dairy cow performance.

Silage properties	Grass silage		s.e.d.[1]	*P*-value
	Control	Spoiled		Trt[2]
Chemistry (g/kg DM)				
Dry matter (g/kg)	302	364	20.5	0.016
Crude protein	203	219	5.30	0.005
Neutral detergent fibre	393	367	8.21	0.004
Ash	13.8	14.0	2.44	0.942
Lactic acid	60.6	67.0	3.37	0.094
Acetic acid	37.1	20.8	3.23	< 0.001
Propionic acid	1.63	0.910	0.286	0.036
Butyric acid	0.079	0.103	0.0235	0.340
Isobutyric acid	< 0.070	< 0.070	-	-
Valeric acid	0.079	0.103	0.0235	0.340
Isovaleric acid	0.145	0.202	0.0387	0.182
Ethanol	2.97	1.76	0.397	0.016
Propan-1-ol	1.34	0.37	0.374	0.033
Propane-1,2-diol	19.4	2.70	2.330	< 0.001
pH	3.84	3.97	0.063	0.043
Silage mycotoxin load (µg/kg DM)				
Aflatoxin B_1	ND	8.11	-	-
Deoxynivalenol	31.3	50.2	-	0.018
Enniatin A/A_1	21.8	21	-	0.675
Enniatin B/B_1	23.4	11.7	-	0.637
Fusaric acid	19.6	11.3	-	<0.001
Moniliformin	13	7.68	-	0.077
Mycophenolic acid	ND	9.73	-	-
Roquefortine C	ND	10.8	-	-
Risk equivalent quantity	4.94	13.6	-	0.076

[1]Standard error of the differences between means;
[2]Treatment;
ND = not detected by LC MS-MS.

6.4.2. Cow performance and rumen metabolism

6.4.2.1. Cow performance

There was no effect of dietary treatment on DM intake (P = 0.439), or milk yield (P = 0.313), with means of 19.5 kg/d ± 0.932 and 22.8 kg/d ± 0.500, respectively (Table 6-5). There was also no effect on fat (P = 0.877), protein (P = 0.405), casein (P = 0.406) or lactose (P = 0.678) concentration of the milk, with means of 42.5, 33.7, 27.3 and 46.8 g/kg, respectively. There was no effect of treatment on urea (P = 0.787), somatic cell count (P = 0.732) or feed efficiency (P = 0.925), with means of 302 mg/L, 143,000 cells/ml and 1.20, respectively.

There was an effect of time for DM intake (P = 0.002) with a general decrease across the 5 d challenge phase from a mean of 20.6 kg/d at d 1 decreasing to a mean of 17.7 kg/d at d 5. There was also an effect of time on protein concentration of the milk (P = 0.041) with an increase across the five d challenge phase from a mean of 33.6 g/kg at d1 increasing to 34.1 g/kg at d 5. In contrast there was a decrease in lactose (P < 0.001) concentration of the milk across the five d challenge phase from a mean of 47.2 g/kg at d 1 to 46.4 g.kg at d 5. There was an effect of time on somatic cell count and feed efficiency, with an increase from 121000 to 184000 somatic cells per ml, and an increase from a feed efficiency of 1.12 to 1.30 across the 5 d challenge phase. There were no interaction effects seen for DMI, milk yield or milk composition parameters (Table 6-5).

There was no effect of treatment (P = 0.195) or time (P = 0.207) on cow rectal temperature, with a mean of 38.0 °C ± 0.059 (Table 6-5). There was no interaction effect seen for temperature (P = 0.486).

6.4.2.2. Rumen pH and ammonia concentration

There was an effect of treatment on rumen pH (P = 0.047), where *post-hoc* analyses revealed these differences to be between treatments CM and SMB, with a mean pH of 6.31 and 6.16, respectively (Table 6-5). All other dietary treatments were found to be intermediate of these values. There was no effect of treatment on ammonia concentration (P = 0.540) of the rumen across the 5 d challenge phase with a mean of 159 mg/L ± 77.8. There was an effect of time demonstrated for both rumen pH (P = 0.022) and ammonia concentration (P < 0.001) where values decreased from d 1 to d 3 and then increased again from d 3 to d 5. There was no interaction found for rumen pH (P = 0.396) or rumen ammonia concentration (P = 0.605).

6.4.2.3. Rumen VFA concentrations

There was a trend (P = 0.077) for the highest total VFA concentration in the SMB treatment (mean of 126 mM) compared with the CM dietary treatment (mean of 120 mM), but this was not significant (Table 6-5)

There was an effect of time (P = 0.007) on total VFAs where from d 1 to d 3 there was a decrease form a mean concentration of 127 mM to 118 mM and then an increase from 118 mM to 125 mM at d 5.

There was an effect of dietary treatment on acetate concentration (P = 0.011) where *post-hoc* analyses revealed that the CM treatment (mean of 76.3 mM) was significantly lower than the SM (mean of 79.2 mM) and SMB treatments (mean of 80.3 mM), with means for C and S intermediate (Table 6-5

There was also an effect of time (P = 0.013) where mean acetate concentration decreased from 80.8 mM at d 1 to 73.6 mM at d 3 and then increased to 79.8 mM at d 5. Likewise, there was an effect of treatment on mean propionate concentration (P = 0.041) where *post-hoc* analysis revealed a significantly higher mean propionate concentration of 24.3 mM in the rumen of C dietary treatment fed cows, in comparison to cows fed dietary treatment SM, with a mean of 22.4 mM. All other dietary treatments were intermediate. There was an effect of time, with mean concentrations of propionate (P < 0.001) decreasing from a mean of 24.7 mM at d 1 to 21.6 mM at d 3, with an increase to a mean of 23.2 mM at d 5.

Mean butyrate concentrations also demonstrated an effect of dietary treatment (P = 0.009) with *post-hoc* analyses revealing the lowest mean of 15.1 mM significantly different to the highest mean butyrate concentration of 17.0 mM in SMB treated dairy cows (Table 6-5). All other dietary treatments were intermediate. There was no effect of time on mean butyrate concentration (P = 0.134). Isobutyrate concentrations were not affected by dietary treatment (P = 0.404), nor were mean valerate concentrations (P = 0.119) with mean concentrations of 1.14 mM ± 0.049 and 2.05 mM ± 0.416

There was an effect of time on isobutyrate (P < 0.001) and valerate (P = 0.006) mean concentrations with a decrease from 1.19 and 2.29 mM at d 1 to 1.05 mM and 2.09 mM at d 3 and then increased from d 3 to 1.12 and 2.26 mM at d 5, respectively.

There was an effect of dietary treatment on mean isovalerate concentration (P < 0.001; Table 6-5). *Post-hoc* analyses revealed that cows treated with C and CM diets had higher mean concentrations of 2.06 and 1.98 mM, respectively, and lower mean concentrations of 1.65,

1.60 and 1.67 mM were found in cows fed the S, SM and SMB dietary treatments, respectively. There was also an effect of time on mean isovalerate concentration ($P < 0.001$) with a decrease from a mean of 1.96 mM at d 1 to 1.65 mM at d 3, followed by an increase to d 5 with a mean of 1.77 mM. There was no interaction effect demonstrated for total VFA concentration ($P = 0.435$) or for any other mean concentrations of individual VFAs (all $P > 0.005$).

6.4.2.4. Rumen VFA proportions

There was no effect of dietary treatment demonstrated on acetate ($P = 0.130$), or valerate ($P = 0.294$) as a percentage of total VFAs with means of 63.9 % ± 0.458 and 1.79 % ± 0.053, respectively (Table 6-5). However, there was an effect of treatment on propionate ($P < 0.001$) and butyrate ($P = 0.002$) as a percentage of total VFAs. For cows fed dietary treatments S and SM, *post-hoc* analyses demonstrated the lowest mean percentage of propionate of 18.1 and 18.2 %, significantly lower than cows fed the C and CM dietary treatments with means of 19.7 and 19.4 %, respectively. Cows fed the SMB diet were intermediate with a mean value of 18.6 % for propionate as a % of total VFAs. There were higher mean percentages of 13.3, 13.4 and 13.5 % of butyrate in cows fed S, SM and SMB dietary treatments, identified as significantly higher than the means of 12.5 and 12.6 % of total VFAs in cows fed the C and CM dietary treatments, respectively.

Isobutyrate and isovalerate as a % of total VFAs also demonstrated an effect of treatment ($P = 0.026$ and $P = < 0.001$, respectively), with cows fed the C and CM treatments revealing a higher means for isobutyrate (0.957 and 0.934 % respectively) and also for isovalerate (1.69 and 1.66 % respectively; (Table 6-5).

In comparison cows fed S, SM and SMB diets demonstrated lower means of 0.887, 0.879 and 0.844 % respectively, for butyrate as a % of total VFAs, and 1.32, 1.29 and 1.33 % respectively, for isovalerate as a % of total VFAs. There were effects of time ($P < 0.05$) for all VFAs as a % of total VFAs (except for valerate), where proportions decreased from d 1 to d 3 before increasing again from d 3 to d 5.

Ratios of acetate to propionate were affected by treatment ($P = 0.004$) where the lowest mean ratio was demonstrated by cows fed the C diet, with a value of 3.23 (Table 6-5). *Post-hoc* analyses revealed this was different to the cows fed dietary treatment S, with the highest mean ratio of 3.60. Cows fed the SMB diets demonstrated an intermediate acetate to propionate ratio of 3.47. Cows fed dietary treatment CM demonstrated a significantly lower acetate to propionate ratio of 3.30, compared to cows fed treatment S (3.60) but not SM, C, CM or SMB.

Similarly, there was an effect of treatment on acetate + butyrate to propionate ratio with the lowest mean ratio demonstrated by cows fed the C diet, with a value of 3.87. *Post-hoc* analyses revealed this was different to cows fed dietary treatment S, SM and SMB, but not CM. Mean values of 4.33 and 4.31 for S and SM treated cows were significantly higher than C and CM (3.95) treated cows, but there was no difference found between SMB (4.20) and CM treated cows. There was an effect of time for both ratios ($P < 0.001$), where again from d 1 to d 3 there was a decrease in ratio value and an increase from d 3 to d 5. There was no interaction effect demonstrated for either acetate to propionate ($P = 0.520$) or acetate + butyrate to propionate ratio ($P = 0.301$).

Table 6-5. Dry matter intake, milk yield and composition, and rumen metabolism parameters of dairy cows exposed to a five-day mycotoxin challenge phase, to assess the impact on the rumen metabolism and microbiome and subsequent effect on performance.

	Dietary treatment[1]					s.e.d[6]	*P*-value		
	C	CM	S	SM	SMB		Trt[7]	d[8]	Trt x d[9]
*Cow performance**									
DM intake (kg/d)	18.1	19.1	19.5	20.1	20.5	1.55	0.439	0.002	0.334
Milk yield (kg/d)	22.4	22.3	22.9	23.1	23.5	0.839	0.313	0.765	0.816
Fat (g/kg)	42.1	42.8	42.4	42.6	42.1	1.26	0.877	0.184	0.818
Protein (g/kg)	34.2	32.8	33.7	34.1	34.4	0.811	0.405	0.041	0.062
Casein (g/kg)	27.6	26.8	27.4	27.6	27.8	0.511	0.406	0.173	0.065
Lactose (g/kg)	47.1	46.7	46.9	46.6	47.0	0.465	0.678	< 0.001	0.735
Urea (mg/L)	297	300	313	300	299	16.8	0.787	0.104	0.793
SCC (x1000/ml)	119	138	166	142	157	55.4	0.732	0.034	0.625
Feed efficiency[2]	1.17	1.22	1.23	1.17	1.16	0.130	0.925	0.009	0.502
Rectal temp. °C	38.1	37.9	38.0	38.0	38.0	0.120	0.195	0.207	0.486
Rumen metabolism									
Rumen pH	6.27	6.31	6.20	6.24	6.16	0.080	0.047	0.022	0.396
Ammonia (mg/L)	205	184	191	195	177	23.7	0.540	< 0.001	0.605
Total VFAs (mM)	123	120	124	123	126	5.22	0.077	0.007	0.435
Acetate	77.6	76.3	80.3	79.2	80.3	3.23	0.011	0.013	0.439
Propionate	24.3	23.2	22.5	22.4	23.4	1.25	0.041	< 0.001	0.252
Butyrate	15.4	15.1	16.6	16.5	17.0	0.893	0.009	0.134	0.586
Isobutyrate	1.17	1.12	1.11	1.09	1.11	0.064	0.404	< 0.001	0.197
Valerate	2.19	2.06	2.28	2.26	2.30	0.143	0.119	0.006	0.307
Isovalerate	2.06	1.98	1.65	1.60	1.67	0.128	<0.001	<0.001	0.132
As a % of total VFAs									
Acetate	63.3	63.7	64.5	64.4	63.9	0.633	0.130	0.040	0.896
Propionate	19.7	19.4	18.1	18.2	18.6	0.523	< 0.001	< 0.001	0.388
Butyrate	12.5	12.6	13.3	13.4	13.5	0.337	0.002	< 0.001	0.003
Isobutyrate	0.957	0.934	0.887	0.879	0.884	0.034	0.026	< 0.001	0.207
Valerate	1.79	1.72	1.83	1.83	1.83	0.078	0.294	0.305	0.653
Isovalerate	1.69	1.66	1.32	1.29	1.33	0.077	< 0.001	< 0.001	0.199
Ac[3] : Prop[4]	3.23	3.30	3.60	3.56	3.47	0.125	0.004	< 0.001	0.520
Ac + But[5] : Prop	3.87	3.95	4.33	4.31	4.20	0.148	0.001	< 0.001	0.301

*Corrected with a co-variate at d 17 of the treatment period.
[1]C = Control silage, S = Spoiled silage, CM = Control silage + mycophenolic acid at 5000 µg/kg DM TMR, SM = Spoiled silage + mycophenolic acid at 5000 µg/kg DM TMR, SMB = Spoiled silage + mycophenolic acid at 5000 µg/kg DM TMR + 150 g/head/d mycotoxin binder. SCC = somatic cell count,
[2]Feed efficiency determined by milk yield (kg/d) / DM intake (kg/d)
[3]Ac = acetate, [4]Prop = propionate, [5]But = butyrate
[6]Standard error of the difference between means
[7]Trt: Treatment
[8]d: Challenge phase day
[9]Trt x d: Interaction between treatment and day along the challenge phase.

6.4.3. Rumen microbial community

6.4.3.1. Main phyla identified

The main phyla identified in the rumen microbiome were Bacteroidetes and Firmicutes that comprised 57 % and 22 % of the total sequence data. Other phyla included, Euryarchaeota at 7 %, and Proteobacteria that made up 4 % of the total sequence data, as did Spirochaetes. Fibrobacteres formed 2 % of the total sequence data, and 1 % each were comprised of Tenericutes, Patescibacteria and Cyanobacteria.

6.4.3.2. Diversity of the rumen microbiome

There was no effect of treatment on any of the four alpha diversity metrics of OBS (P = 0.785), Chao1 (P = 0.973), Shannon (P = 0.292) or Inverse Simpson (P = 0.296; Table 6-7). Archaea to Bacteria ratios did not differ significantly between cows for any of the treatment diets fed (P = 0.843) with a mean value of 0.078 ± 0.006.

The OTUs identified as significantly (LDA ≥ 2.0 or ≤ -2.0) higher or lower in relative abundance between cows fed different treatment diets are presented in Table 6-8. There was an effect of the addition of MPA on the abundance of *Methanobrevibacter* identified as species *Methanobrevibacter smithii* with 99 % identity, where the addition of MPA increased abundance from 1369 in C fed cows to 1687 in CM fed cows (LDA = 2.22), and from 1505 in S fed cows to 1818 in SM fed cows (LDA = 2.20). In contrast two *Prevotellaceae* species, OTU0004 (LDA = -2.18) and OTU0028 (LDA = -2.02) decreased in relative abundance when MPA was added, with values of 1043 and 329 respectively in C diet fed cows, decreasing to 745 and 121 respectively in CM diet fed cows. An unclassified species of the Bacteroidales order increased in relative abundance from 365 to 1465 when cows received CM diets in comparison to C (LDA = 2.74). Similarly, an *Acidaminococcaceae* species, with 95 % identity as *Succiniclasticum ruminis,* increased from a mean abundance of 2984 to 3368 when cows were fed SM in comparison to S diets.

An effect of silage type was identified for the relative abundance of the unclassified species of the Bacteroidales order, with an increase from 365 to 731 in S fed cows compared with C fed cows (LDA = 2.27; Table 6-8). Similarly, an increase in abundance from 2966 in CM fed cows to 3368 in SM fed cows was identified for *Succiniclasticum ruminis* (LDA = 2.31), and an increase from 745 in CM fed cows to a mean abundance of 972 in SM fed cows for a species of *Prevotellaceae* (LDA = 2.06).

There was an effect of the mycotoxin binder found on the relative abundance of *Succiniclasticum* (95 % identity) , *Methanobrevibacter smithii* and a third *Prevotellaceae* species identified at 96 % as *Prevotella ruminicola* (Table 6-8). Addition of a binder resulted in a decrease in relative abundance of these three OTUs from 3368 to 2718 in *Succiniclasticum ruminis* (LDA = 2.51), from 1818 to 1322 in *Methanobrevibacter smithii* (LDA = 2.40), and from 891 to 615 in *Prevotella ruminicola* (LDA = 2.14).

Table 6-6. The main phyla as a % of total sequence data of the rumen microbial composition, in dairy cows exposed to a five-day mycotoxin challenge phase, to assess the impact on the rumen metabolism and microbiome and subsequent effect on performance.

Main phyla identified with 16S rRNA primers	% of total sequence data
Bacteroidetes	57%
Firmicutes	22%
Euryarchaeota	7%
Proteobacteria	4%
Spirochaetes	4%
Fibrobacteres	2%
Tenericutes	1%
Patescibacteria	1%
Cyanobacteria	1%
Chloroflexi	<1%
Actinobacteria	<1%
Planctomycetes	<1%
Elusimicrobia	<1%
Epsilonbacteraeota	<1%
Synergistetes	<1%

Table 6-7. Alpha diversity metrics for the rumen microbial composition, in dairy cows exposed to a five-day mycotoxin challenge phase, to assess the impact on the rumen metabolism and microbiome and subsequent effect on performance.

Alpha diversity metrics	Dietary treatment[1]					s.e.d.[2]	*P*-Value
	C	CM	S	SM	SMB		Trt[3]
OBS	1365	1362	1362	1378	1374	37.4	0.785
Chao1	1825	1833	1869	1806	1842	121.7	0.973
Shannon	4.95	4.91	4.91	4.95	5.03	0.090	0.292
Inv. Simpson	36.7	35.3	26.2	37.1	41.7	4.88	0.296
Archaea : Bacteria ratio	0.069	0.084	0.078	0.082	0.076	0.023	0.843

[1]C = Control silage, S = Spoiled silage, CM = Control silage + mycophenolic acid at 5000 µg/kg DM TMR, SM = Spoiled silage + mycophenolic acid at 5000 µg/kg DM TMR, SMB = Spoiled silage + mycophenolic acid at 5000 µg/kg DM TMR + 150 g/head/d mycotoxin binder.
[2]Standard error of the difference between means
[3]Trt: Treatment

Table 6-8. Linear discriminant effect size analysis of mean filtered sequence counts for significant OTUs (LDA ≥ 2.0 or ≤ - 2.0) identified in the rumen microbiome in dairy cows exposed to a five-day mycotoxin challenge phase, to assess the impact on the rumen metabolism and microbiome and subsequent effect on performance.

Comparison	OTU[1]	Dietary treatment[2]					LDA[3]	SILVA 123 SEED[4]	BLASTn Type Strain[5]	% Ident[6]
		C	CM	S	SM	SMB				
Effect of mycophenolic acid addition	OTU0003	1369	1697				2.22	*Methanobacteriaceae*	*Methanobrevibacter smithii* ATCC 35061	99
	OTU0004	1043	745				-2.18	*Prevotellaceae*	*Prevotella copri* DSM 18205	93
	OTU0005	365	1465				2.74	*Bacteroidales* (unclassified)	*Duncaniella freteri* strain TLL-A3	86
	OTU0028	329	121				-2.02	*Prevotellaceae*	*Prevotella ruminicola* strain Bryant 23	91
	OTU0001			2984	3368		2.29	*Acidaminococcaceae*	*Succiniclasticum ruminis* strain SE10	95
	OTU0003			1505	1818		2.20	*Methanobacteriaceae*	*Methanobrevibacter smithii* ATCC 35061	99
Effect of silage	OTU0005	365		731			2.27	*Bacteroidales* (unclassified)	*Duncaniella freteri* strain TLL-A3	86
	OTU0001		2966		3368		2.31	*Acidaminococcaceae*	*Succiniclasticum ruminis* strain SE10	95
	OTU0004		745		972		2.06	*Prevotellaceae*	*Prevotella copri* DSM 18205	93
Effect of binder	OTU0001				3368	2718	2.51	*Acidaminococcaceae*	*Succiniclasticum ruminis* strain SE10	95
	OTU0003				1818	1322	2.40	*Methanobacteriaceae*	*Methanobrevibacter smithii* ATCC 35061	99
	OTU0006				891	615	2.14	*Prevotellaceae*	*Prevotella ruminicola* strain Bryant 23	96

[1] Order of taxonomic units sequence identification number.
[2] C = Control silage, S = Spoiled silage, CM = Control silage + mycophenolic acid at 5000 µg/kg DM TMR, SM = Spoiled silage + mycophenolic acid at 5000 µg/kg DM TMR, SMB = Spoiled silage + mycophenolic acid at 5000 µg/kg DM TMR + 150 g/head/d mycotoxin binder.
[3] Linear discriminant analysis value. A value > 2.0 denotes a significant difference in relative abundance between dietary treatments.
[4] Families identified from matches on the SILVA 123 SEED database
[5] Species identified from matches blasted using the BLASTn tool provided by NCBI.
[6] Percentage identity match between the sequence blasted using the BLASTn tool, and the most similar reference sequence hit. As percentage identity approaches 100%, the more similar the sample sequence to the reference sequence.

6.5. Discussion

6.5.1. Silage production and chemistry

The protocol to spoil the grass silage was based on previous work by Snelling et al., (2023) and (Wilkinson and Davies, 2013), in which delayed sealing and poor compaction was suggested to lead to a slower fermentation and greater spoilage. However, there was no evidence from the silage chemistry that the spoiled grass silage experienced a poorer fermentation in comparison to the control - there were no differences in lactic acid concentration between the silages and pH was below the theoretical pH maximum value determined by Haigh (1987) for both silages (Equation 12). Acetic acid concentration did differ, suggesting a higher proportion of obligate heterofermentative LAB activity during the fermentation in the control silage; supported by a greater concentration of propane-1,2-diol and ethanol in the control silages in comparison to the spoiled (Elferink et al., 2000; S. J.W.H. Oude Elferink et al., 2001). Despite the differences in the fermentation of the silages, the DM content of both silages were toward the higher end in comparison to grass silages surveyed in Chapter 3 and as DM was found most strongly negatively correlated with mycotoxin content in comparison to the other fermentation parameters, this may explain why mycotoxin content of this silage did not reflect that seen on farms in Great Britain.

Moreover, despite a wider range of mycotoxins found in the spoiled grass silage clamps, and statistical differences found, it could be argued biologically insignificant as levels between the two silages were within the same magnitude. The REQ value for both silages of 4.94 µg/kg DM and 13.6 µg/kg DM would be classified as lower risk (< 50 µg/kg DM) for adverse effects to dairy cow health or performance, before the addition of 5000 µg/kg MPA, which would raise the risk to high (> 150 µg/kg DM). The levels of endogenous mycotoxins in the spoiled silage could therefore be considered negligible in effects on the dairy cow when administered alone, but it may be that in combination with one another there is a possibility of synergistic effects (Speijers and Speijers, 2004). Furthermore, it was surprising that aflatoxin B_1 was identified, albeit at low levels, in the grass silage as this is not reported widely in the literature and was not present in any of the grass silage samples in Chapter 3. Overall, any effect of mycotoxin contamination on cow performance or rumen metabolism and the microbiome in this study is likely to be as a result of the addition of MPA.

As this study was carried out previous to the work in Chapter 3 and 4, the correlation between fermentation parameters and mycotoxin contamination had not yet been identified. Future studies that aim to form spoiled silage should manipulate these fermentation parameters in order to increase endogenous mycotoxin production in the spoiled silage. It is important

however that the silage does not experience excessive spoilage that it becomes unpalatable for the cow, as this will affect DM intake and subsequent milk yield (Grant and Ferraretto, 2018). High acetic acid levels in silage have in particular, been responsible for decreased DM intake in cows (Gerlach et al., 2021). Any impacts on performance of the cow, may mask any mycotoxin treatment effects, confounding the experiment.

6.5.2. Animal performance and health

Mycophenolic acid despite being identified in only 2 samples of silage sampled in Chapter 3, has been identified by others as a prominent grass silage mycotoxin (Penagos-Tabares et al., 2023; Schneweis et al., 2000). Associated with *Penicillium*, MPA is a potent immunosuppressive agent (Bentley, 2000) used in human medicine, and so was not costly to administer in the quantities required to adequately dose dairy cow diets. As there are no studies to the author's knowledge, that have evaluated the effect of MPA on cattle, cow temperatures were measured daily and feed intake monitored closely for any adverse effects of the mycotoxin on cow health. Additionally, treatment diet exposure was limited to a five day duration. Over the five challenge phases there were no indications that cow health was adversely affected during exposure to the treatment diets. Considering that the cows in this study consumed approximately 12.5 kg of silage DM per day (64 % of 19.5 total kg DM TMR), the additional MPA would result in a proportional silage concentration of approximately 3,150 µg/kg DM TMR, or an intake of 32 mg of MPA per day - well within the magnitude of MPA found in grass silages previously by Penagos-Tabares et al., (2024) with a mean concentration of 2,530 µg/kg, equivalent to an intake of 25 mg of MPA per day.

DM intake measured over the 5 d challenge phase was unaffected by dietary treatment, in contrast to Custodio et al., (2020) whom observed a 0.6 kg/d drop in DM intake when a mycotoxin dosed TMR was fed to beef cattle compared with the control TMR. However Dänicke et al., (2005) found no effect of an increasing DON concentration of wheat from 0.0 mg/kg DM to 5.0 mg/kg DM on the DM intake of dairy cows and studies by Charmley et al., (1993); Dänicke et al., (2014); Kinoshita et al., (2018); and Korosteleva et al., (2009), all compared the DM intake of cows with combinations of ZEA and DON at concentrations up to 12mg/kg DM with no effect on DMI, despite feeding treatment diets for up to 189 days (Kinoshita et al., 2018). It is therefore an important to note the contamination status of a feed cannot be interpreted from the DMI by cattle.

Milk yield over the short-term feeding period in the current study was unaffected by treatment, which is consistent with the lack of change in DMI by the cows in this study. Studies that have supplemented dairy cow diets with DON (Charmley et al., 1993), aflatoxins (Ogunade et al.,

2016) or *Fusarium* mycotoxins (McKay et al., 2019) for longer periods of time than the current study, also did not observe any impact of mycotoxin consumption on the milk yield. Milk fat and protein concentration were also unaffected by dietary treatment in the current study, despite differences in the ratio of rumen acetate to propionate observed.

6.5.3. Rumen metabolism

Despite the MPA content of the silages, there was no effect on DM intake, or milk yield over the 5 d challenge phase. There was also no effect on milk composition or feed efficiency either, suggesting that over a five day feeding challenge MPA at 5000 µg/kg DM TMR there was no negative impact on animal performance. As alterations to VFA proportions were demonstrated with MPA addition a longer duration of exposure may lead to eventual changes in milk composition. Rectal temperature is routinely used as a measure of cow health (Burfeind et al., 2010; Smith and Risco, 2005) and did not differ across the dietary treatments fed indicating, no immediate adverse effects on cow health induced by a 5 d feeding challenge at 5000 µg/kg DM TMR.

Rumen pH was altered by dietary treatment with the lowest pH of 6.16 observed in cows fed SMB diets, though all mean values were found within expected ranges (Membrive, 2016). Cows were therefore unlikely to be experiencing any metabolic dysbiosis such as sub-acute ruminal acidosis, demonstrated to alter the ability of rumen organisms to function efficiently. This may have been as a result of a higher total VFA concentration observed for cows fed SMB diets, that demonstrated a trend ($P = 0.077$). The mycotoxin binder may have had a stimulatory effect on the production of VFAs in the rumen. The addition of yeast has previously been demonstrated by Wang et al., (2023) to increase rumen total VFA concentration, when fed to sheep, but whether this effect would be seen in dairy cows with a yeast cell wall – derived product, is unknown. Rumen ammonia production was not affected by the treatment diets fed, suggesting that there was no large shift in the rumen microbial composition (Eschenlauer et al., 2002) towards a fermentation that would be nutritionally inefficient for the dairy cow. Further supporting the lack of effect of dietary treatment on animal performance.

Acetate to propionate ratio was expected to be higher in the groups that had received the control silage as part of the dietary treatment due to the higher fibre content of the control grass silage, however this was not seen suggesting a change in rumen microbial composition as of a result of other interacting factors than the fermentation profile of the silage alone. (Davis, 1967; Sutton et al., 2003). In Chapter 5, when dosed with the same concentration of MPA, there were no alterations seen on the ammonia concentration, VFAs produced, or pH of the rumen.

6.5.4. Rumen microbial composition

The 16S rRNA gene sequence data comprised in total 787,712 reads after quality filtering that were assigned to a total of 2,873 features, of which 1112 could be assigned up to genus level with 97 % identity. The main phyla identified were as expected with Firmicutes and Bacteroidetes comprising the majority, and in agreement with the findings of Henderson et al. (2015) and Hartinger et al. (2022). The LEfSe revealed changes to the relative abundance of individual taxa, namely *Methanobrevibacter smithii*, *Succiniclasticum ruminis* and multiple *Prevotella* species as a result of MPA addition, silage type and the inclusion of a mycotoxin binder. Functional activity may be inferred from a high confidence classification of some OTUs identified in this study, such as *Succiniclasticum ruminis* and *Methanobrevibacter smithii* (Miller et al., 1982).

Succiniclasticum species are of the Acidaminococcaceae, (Phylum: Firmicutes), and have been demonstrated to utilise succinate as a substrate solely, with fermentation products of acetate, propionate and butyrate (Seshadri et al., 2018), however *S. ruminis* has only been demonstrated to yield propionate (van Gylswyk, 1995). The relative abundance was found to increase when spoiled silage was fed in comparison to the control and even more so when MPA was added. In contrast upon addition of the binder to the SM diet, relative abundance of *S. ruminis* decreased, to lower than that of the control or spoiled silage with MPA. Additionally, *Succiniclasticum ruminis* was found to be in highest relative abundance in the rumen of cows fed SM, whilst rumen propionate proportions were also the lowest. This is contradictory to the expectation of a greater rumen propionate concentration with an increase in the relative abundance of *Succiniclasticum* species (van Gylswyk, 1995). As *Succiniclasticum ruminis* is a specialist with regards to requiring succinate provision for growth, the population may be more sensitive to changes in rumen fermentation as a result of differing silage chemistry or addition of MPA. Succinate concentration in the rumen was not measured in the study, but as it can be utilised by other species of Acidaminococcaceae and Veillonellaceae (Seshadri et al., 2018), being readily converted to propionate (Blackburn and Hungate, 1963), it may have been that other microbial groups decreased in relative abundance, providing a greater proportion of succinate for metabolism by *S. ruminis*.

Methanobrevibacter smithii, is a hydrogenotrophic methanogenic archaea of Methanobacteriaceae (Phylum: Euryarchaeota) that is known to form a symbiosis with the rumen protozoa (Seshadri et al., 2018). With the addition of MPA in both control and spoiled silage, relative abundance was increased, suggesting a stimulatory effect of MPA on methanogen growth, and even protozoal abundance. This was unexpected as eukaryotic cells

are reported to be sensitive to the effect of MPA as it can disrupt the process of DNA synthesis (Freedman et al., 2020), though there is little information regarding the sensitivity of prokaryotic cells. Additionally, though not MPA, Hartinger et al. (2023) observed an increase in protozoal abundance after ZEA was administered to dairy cows fed a forage rich diet, but this was most likely as a result of the degradation ability of protozoa on ZEA (Westlake et al., 1989). Effects of protozoal degradation of MPA have not yet been elucidated but other fungal species and some bacteria have been demonstrated to metabolise MPA (Jones et al., 1970), though these are not of rumen origin.

Silage type did not result in a significant difference in relative abundance of *Methanobrevibacter* species however the addition of the mycotoxin binder, reduced relative abundance to below that which was seen for all other dietary treatments. This suggested the binder demonstrated an inhibitory effect on *M. smithii* whether directly or indirectly. Whether this effect extended to a subsequent decrease in methanogenic activity also, is unknown. Acetate to propionate ratio of the rumen fermentation for SMB fed cows were intermediate in comparison to other treatment diets, suggesting that relative abundance was not altered as a result of a shift in a higher proportion of propionate that would be a competitive pathway for the utilisation of H_2 produced (Moss et al., 2000). Methane output was not investigated in this study, and so the methanogenic activity as a result of changes in relative abundance of *Methanobrevibacter smithii*, cannot be conclusively determined. Archaea : Bacteria ratios can be used to identify shifts in microbial populations associated with methanogenesis (Wallace et al., 2014) however no effect of treatment diet were found for the archaea : bacteria ratios in this study.

For OTUs classified as species of the genus *Prevotella*, interpreting functional impact from changes in relative abundance is difficult due to the association of *Prevotella* with many different metabolic functions within the rumen. Prevotellaceae, can utilise xylose, pectin, starch, and protein and end products of their fermentation include acetate, propionate, and succinate (Accetto and Avguštin, 2019; Betancur-Murillo et al., 2023). Additionally, the mixed responses in population abundances of the *Prevotella* species to the different treatment diets only reiterates the difficulty of interpreting the function of this extremely diverse genus (Accetto and Avguštin, 2019). The low confidence species identification of OTU0005 (86%), a member of *Bacteriodales* is most likely a novel and uncharacterised *Prevotella* species, rather than *Duncaniella freteri* strain TLL-A3 which has been isolated from faecal pellets of mice (Miyake et al., 2020).

Future work should look to incorporate metagenomic methods to understand the implications of these small scale changes in the relative abundance of low abundance OTUs in the wider context of the full rumen microbiome. For example, the incorporation of the protozoal abundance would aid in understanding the shifts in abundance of *Methanobrevibacter* species. There may even be opportunities for investigating changes in rumen methane outputs with regards to the incorporation of the mycotoxin binder; studies have established a relationship between reduced methane outputs when live yeasts are included in feed (Chung et al., 2011; McGinn et al., 2004).

6.5.5. Conclusions

In conclusion, feeding grass silage with or without additional MPA and the inclusion of a mycotoxin binder, was demonstrated to alter VFA proportions in the rumen, without effect on overall rumen microbial composition, and alterations in abundance of individual OTUs. Despite no effect of dietary treatment on milk yield, protein or fat content, VFA production was altered as a result, indicating that there may be underlying interactions between the spoiled silage with the microbiome, that could present changes to animal performance, given a longer duration of feeding. The inclusion of a mycotoxin binder resulted in a lower mean rumen pH and a tendency for a higher total VFA concentration, as well as a reduction in *Methanobrevibacter smithii*. Lack of an effect of mycotoxin consumption on the performance of the cows limited the ability to evaluate the efficacy of the binder.

Chapter 7

General discussion

7. General discussion

Grass silage mycotoxins have been largely understudied and some of the most commonly occurring mycotoxins in grass silages identified in Chapter 3, (i.e. PEN and FUS) are currently not regulated by the EU, nor are there any advisory limits provided, despite them possessing properties that can impact on the rumen fermentation (Chapter 5), with potential to disrupt the performance of dairy cows (Chapter 6; May et al., 2000; Raphael, 1947; Zhang et al., 2021). Grass silage is a major component of feed for cattle in Great Britain and Northern Europe (Skladanka et al., 2013) and it is important that the body of scientific information concerning mycotoxin contamination of grass silage and mitigation is increased substantially if herd health, efficiency and sustainability of the dairy industry is to be improved (Põldaru and Luik-Lindsaar, 2020). The more studies that are carried out on the effects of grass silage mycotoxins on dairy cow performance, the more tailored and therefore reliable, predictions such as the Alltech© REQ, can become.

7.1. Grass silage mycotoxins in Great Britain

This thesis set out to identify the mycotoxin profile of grass silages on commercial farms in Great Britain and any relationships with on-farm management. In Chapter 3, across a selection of 37 farms in Great Britain the mycotoxin content ranged from lower (0.400 µg/kg DM) to higher risk (8022 µg/kg DM) according to the REQ (Figure 7-1) without evidence of substantially poor compaction, butyric fermentation or excessive visible mould overall. Moreover, the study demonstrated that the fermentation profile of the silage, namely a lower DM content, higher lactic and acetic acid concentration and higher ethanol content can increase the resultant REQ of the silage produced. This was surprising as acetic acid and ethanol have both been demonstrated to inhibit fungal growth (Alcano et al., 2016; Rogawansamy et al., 2015) and improve aerobic stability of silage (Danner et al., 2003) suggesting the synthesis of mycotoxins may be triggered by the initial fermentation stages as well as upon air exposure at clamp opening. These findings also strongly reiterate that there is no correlation between the presence of fungi and the production of mycotoxins as has been demonstrated by others (Franco et al., 2021; Manni et al., 2022). The silage additive and inoculant industry should aim to understand how certain products that manipulate the initial fermentation may also therefore inadvertently impact on mycotoxin production.

Additionally, the findings from Chapter 3 highlighted the need for more regular mycotoxin testing on-farm, as there were no obvious indications to a farmer at feed-out that a silage may be higher risk for adverse mycotoxin impacts on animal performance. This was demonstrated also by the lack of change in DMI in Chapter 6 by cows when fed a grass silage TMR with

5000 ug/kg DM TMR of an immunosuppressive mycotoxin MPA. No change in cow DMI or palatability, with a grass silage without visible signs of mould and demonstration of a high lactic acid fermentation, is highly likely to pass unnoticed by a farmer yet could be a possible source of mycotoxin contamination for the herd. It is common for silages made to be bought and sold between farms, without mycotoxin testing ever occurring. Not only posing a risk to animal health and performance, but there may be an issue of fungal cross contamination on farm, though this has not yet been studied. Farm hygiene with respect to mycotoxin production remains an interesting avenue for exploration.

Another point to support more frequent testing for on-farm silage production is that the mycotoxin profile of grass silage can change throughout time. In Chapter 4, the mini silo study demonstrated that DON, enniatin B_1 and FUS entered into the silo with the fresh cut grass and were then found to be either absent (FUS), at a similar value (enniatin B_1) or increased (DON) in concentration after the ensiling phase. Upon aerobic exposure DON decreased in concentration. Furthermore, other mycotoxins appeared during the fermentation period: FUM, ergotamine(in)e and PEN and after a period of aerobic exposure roquefortine C, moniliformin, and MPA appeared. As mycotoxins are physically stable compounds (Kabak, 2009; Liu et al., 2024) , it suggests their disappearance is due to breakdown by other fungal or bacterial species present in the silage (Gourama and Bullerman, 1995; Sadiq et al., 2019). With the association of higher lactic and acetic acid concentration with higher REQ, there is an interesting opportunity for the engineering of a silage inoculant with specific anti-mycotoxigenic LAB strains whilst still providing adequate lactic acid fermentation to inhibit other spoilage organisms.

The REQ value of the silage increased across the ensiling timepoint as the mycotoxin profile of the silages increased in diversity. However, the REQ value did not rise above that of the lower risk category in the mini silo studies (Figure 7-1), and it could be that lack of any environmental impact (particularly rain, for example) meant that the mini silo management, and environmental conditions experienced were more “sterile” than of that on-farm. The environmental impact of weather (Skladanka et al., 2013), and poor field conditions (Hodulíková et al., 2016) could be interactive factors with the fermentation and management of the silage that are yet to be meticulously studied with regards to the mycotoxin content of grass silage.

There are, however, limitations to estimating the overall mycotoxin content of a silage (McElhinney et al., 2016b). Firstly, sampling location can impact on the result provided and can lead to a large over or underestimate of the whole clamp from a single localised area. As

laboratory mycotoxin testing can be costly, a bulked sample from different areas of the clamp is often the best compromise between providing a representative sample, without the associated laboratory cost (McElhinney et al., 2016b). On-site rapid tests such as lateral flow devices, can be a cheaper option on-farm, than laboratory testing, but results are less accurate, and the technology for grass silage multiple mycotoxin testing is not yet developed (Maragos and Busman, 2010). As demonstrated in Chapter 5, the combinations of mycotoxins present are an important factor to incorporate into any risk assessment of mycotoxin exposure. In Chapter 3 of this thesis, grass silage samples were obtained from coring into the clamp face at four sites located in areas that were expected to have been most variable to each other in terms of the cut, compaction density and consequent rate of aerobic exposure, to increase the applicability of the result to the variability seen over the clamp. It is however important to note therefore, that the estimated mycotoxin profile for any silage or feed is only reflective of the content at the moment of sampling as supported by Chapter 4.

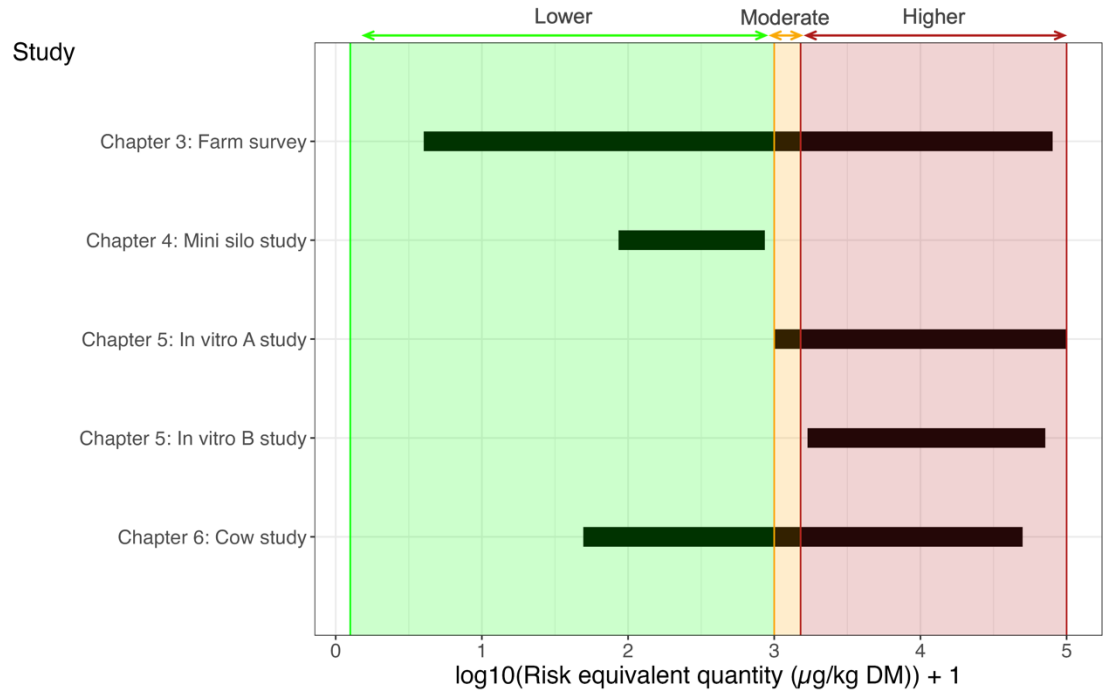


Figure 7-1. The log_{10} risk equivalent quantity (+1) determined by Alltech©, for mycotoxin content either as a treatment addition or endogenously formed within silage, for the experimental studies in each chapter of this thesis. The shading represents the risk categories to the performance of a dairy cow, where green represents a lower risk, orange represents a moderate risk, and red represents a higher risk to animal performance and health.

7.2. The effect of grass silage mycotoxins on rumen fermentation, the rumen microbiome and performance in dairy cows

A series of *in-vitro* studies were carried out in Chapter 5 that identified the importance of considering interaction effects of mycotoxins when assessing their impact on rumen fermentation (Fink-Gremmels, 2008). At higher risk REQ levels, when administered individually, there were no effects observed on the VFAs produced in comparison to the control cultures. However, when combinations of mycotoxins were introduced, at levels that were considered higher risk (Figure 7-1), but within ranges found on GB farms, effects on the concentrations of total VFAs, propionate and butyrate were demonstrated. Though relative proportions of VFAs were not altered, the study demonstrated that combinations rather than increased individual concentrations can impact on rumen fermentation. Though the concept of synergistic, additive or even antagonistic effects of mycotoxins in combination is not novel (Grenier and Oswald, 2011), there are extremely limited studies on grass silage mycotoxins. This highlights the necessity for combinations of mycotoxins to be considered in EU or FAO

regulatory guidelines, rather than limits calculated on single mycotoxin dosages. Particularly any future experiments designed to assess the impact on rumen fermentation should consider the inclusion of multiple mycotoxins as they appear in natural contamination.

Therefore, a large limitation being that in the final study (Chapter 6) a contaminated grass silage representative of at least the median REQ observed in Chapter 3 (295 µg/kg DM), was unable to be produced for 5 x 5 d feeding challenges. Though the addition of 5000 µg/kg DM TMR of MPA was sufficient to raise the REQ of the treatment diets to the category of higher risk from lower risk (Figure 7-1), it meant that effects seen were likely as a result of MPA addition alone. MPA addition on its own at levels five times that included in the cow study, had already demonstrated no impact on rumen fermentation parameters *in-vitro* (Chapter 5). Though there were in fact alterations in VFA proportions when MPA was administered *in-vivo*, there was no subsequent effect on cow performance, milk yield or any alterations to milk composition demonstrated over the study period. The rumen microbiome is currently thought to return to a relatively stable state after a short-term moderate change, for example a change in the diet (Costa-Roura et al., 2022), or upon encountering plant toxins (Dominguez-Bello, 1996) and as the challenge period was only for five days, and there were no severe shifts in proportions of VFAs, rumen pH, or DMI, it was unsurprising there were no effects observed on animal performance. Other studies that have observed adverse effects of mycotoxins on animal performance have had periods of exposure that have ranged from 29 d (Batista et al., 2024) to 54 d (Catellani et al., 2023) but in contrast studies that have not demonstrated an effect of mycotoxin on animal performance have included up to 13 w of mycotoxin exposure (Winkler et al., 2014). These studies were, however,r all investigating *Fusarium* mycotoxins and not MPA.

As a result, the mycotoxin mitigation effect of the binder on cow performance could not be assessed as no alterations in performance were observed in Chapter 6. There were changes in relative abundance of *Methanobrevibacter smithii*, as a result of binder addition that may be of interest for further investigation – particularly as *Methanobrevibacter* comprise around 60 % of the archaea that have been sequenced (McSweeney and Mackie, 2012). Methane output was not collected in the study and was beyond the scope of this thesis, but as there is much interest in lowering methane outputs from cattle to improve sustainability (Hook et al., 2010; Min et al., 2022), it provides an interesting avenue for further exploration.

A limitation to understanding the rumen microbiome from amplicon sequence data is that there is a level of taxonomic bias associated with PCR amplifications, and activity of certain organisms cannot be concluded with information on taxonomic abundance alone (Frioux et

al., 2020). Furthermore, there still remains a large part of the rumen microbiota that are unclassified (Henderson et al., 2019), and knowledge on the full range of metabolic activity certain groups carry out is still emerging (Accetto and Avguštin, 2019; Betancur-Murillo et al., 2023). Nevertheless, 16s rRNA sequence data combined with VFA data (Seshadri et al., 2018), and investigating effects on relative proportions of certain groups such as the Archaea : Bacteria ratio (Wallace et al., 2014), have been reported as effective tools for inferring shifts in microbial composition that may correlate with certain overall fermentation activity of the microbiome. However, a previous study by Hartinger et al. (2022) identified a reduction in acetate concentration during ZEA exposure in dairy cows, without subsequent change to the high abundance families of Ruminococcaceae and Lachnospiraceae, despite being fibrolytic associated families of the Firmicutes phylum. This highlights the complexity of inferring rumen fermentation activity from taxonomic abundance and inferences should be made with caution. Still, the identification of feature-level changes, though at small scales in relative abundance, can still provide information on interesting interactions of treatments with some individual taxa, provided there is reference information and % identity is high. In Chapter 6, though no large scale shifts in abundance were observed at the phylum level, there were mixed effects on abundance of individual OTUs with the addition of MPA on *Succiniclasticum ruminis*, *Prevotella* species, and *Methanobrevibacter smithii*. Despite the lack of combined endogenous mycotoxins in the spoiled silage, Chapter 6 demonstrated that during a 5 d exposure period, MPA elicited an effect on the rumen microbial composition.

7.3. Conclusions

This thesis identified prominent non EU regulated mycotoxins of PEN, FUS and regulated mycotoxins of DON in grass silages in Great Britain that may all pose a risk to rumen fermentation and subsequent animal performance. Fermentation parameters of grass silage were found to influence mycotoxin risk, and mycotoxins present in silage changed throughout, highlighting the need for more frequent mycotoxin testing. Prominent grass silage mycotoxins at levels of inclusion that would exceed natural contamination levels demonstrated no effect on rumen fermentation parameters during an *in-vitro* trial, but in combination with one another at levels found in grass silages in Great Britain, demonstrated effects on rumen VFAs such as propionate and butyrate concentration. This could lead to microbial compositional changes in the cow. A grass silage mycotoxin, MPA, demonstrated an effect on rumen fermentation with decreased acetate:propionate ratio and changes in abundance of methanogenic archaea, a protein-utilising bacteria, and a succinate utilising bacteria, but no effect on animal performance was observed for a 5 d duration of exposure.

In conclusion, grass silage mycotoxins demonstrate an ability to alter rumen fermentation when in combination with one another, at moderate levels found commonly in visibly unspoiled grass silage across Great Britain, which could impact on dairy cow performance.

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9. Appendix

Appendix Table 1: The mycotoxin assignment of the forty-two mycotoxins analysed, to the four main fungal genera possessing the ability to synthesise them.

Mycotoxin	Fungal genera			
	Penicillium	*Fusarium*	*Aspergillus*	*Claviceps*
15-acetyl-4-deoxynivalenol		x		
3-acetyldeoxynivalenol		x		
Aflatoxin B_1			x	
Aflatoxin B_2			x	
Aflatoxin G_1			x	
Aflatoxin G_2			x	
Beauvericin		x		
Citreoviridin	x			
Citrinin	x		x	
Cyclopiazonic acid	x			
Deoxynivalenol		x		
Deoxynivalenol-3-glucoside		x		
Enniatin A/A_1		x		
Enniatin B/B_1		x		
Ergocornin(in)e				x
Ergocristin(in)e				x
Ergocryptin(in)e				x
Ergosin(in)e				x
Ergotamin(in)e				x
Fumonisin B_1		x	x	
Fumonisin B_2		x	x	
Fumonisin B_3		x	x	
Fusarenon X		x		
Fusaric acid		x	x	
Gliotoxin	x			
HT2 Toxin		x		
Lysergol				x
Methylergonovine				x
Moniliformin		x		
Mycophenolic acid	x			
Neosolaniol		x		
Nivalenol		x		

Table continued on next page

Appendix Table 1. (continued)

Mycotoxin	Fungal genera			
	Penicillium	*Fusarium*	*Aspergillus*	*Claviceps*
Ochratoxin A	x		x	
Ochratoxin B	x		x	
Patulin	x		x	
Penicillic acid	x		x	
Roquefortine C	x			
Sterigmatocystin			x	
T2 Toxin		x		
Verruculogen	x		x	
Wortmannin	x			
Zearalenone		x		

"x" denotes the ability of species identified in the genera to synthesise the associated mycotoxin

Appendix Table 2. The components of buffer medium B, by Lowe et al., (1985) used to form the culture inoculum for the pilot *in-vitro* experiment involving a consecutive batch culture.

Buffer medium B components	In 1L
Basal medium B solution[1]	810 ml
KH_2PO_4 solution (68g L^{-1})	10 ml
Yeast extract solution (50g L^{-1})	10 ml
Vitamin solution[2]	10 ml
Reducing solution[3]	10 ml
Na_2CO_3 solution (80g L^{-1})	50 ml
Distilled water	100 ml
[1]Basal medium B solution	
KCl	0.60 g
NaCl	0.60 g
$MgSO_4 \cdot 7H_2O$	0.50 g
$CaCl_22H_2O$	0.20 g
NH_4Cl	0.54 g
Trypticase peptone	1.00 g
PIPES Buffer	1.50 g
[4]Coenzyme M solution	10.0 ml
[5]Fatty acid solution	10.0 ml
[6]Trace element solution	10.0 ml
[7]Haemin solution	10.0 ml
Resazurin (1g L^{-1})	1.0 ml

[2]Vitamin solution		
Pyridoxine HCl	Vitamin B_6	0.200 g
Thiamine HCl	Vitamin B_1	0.200 g
Riboflavin	Vitamin B_2	0.200 g
Nicotinamide	Vitamin B_3	0.200 g
Calcium D-pantothenate	Vitamin B_5	0.200 g
p-amino-benzoic acid	Vitamin B_{10}	0.025 g
Cyanocobalamin	Vitamin B_{12}	0.025 g
Folic acid	Vitamin B_9	0.025 g
Biotin	Vitamin B_7	0.025 g
1,4-naphthoquinone	Vitamin K	0.250 g
5mM HEPES Buffer	Make up to 1L	
[3]Reducing solution		
$Na_2S \cdot 9H_2O$		2.5 g
L- Cysteine HCl		2.5 g
[4]Coenzyme M solution		
2-mercaptoethanesulfonic acid (sodium salt)		4.00g
Distilled water	Make up to 1L	
[5]Fatty acid solution		
Acetate		6.85 ml
Propionate		3.00 ml
Butyrate		1.84 ml
2-methyl butyrate		0.55 ml
Iso-butyrate		0.47 ml
Valerate		0.55 ml
Iso-valerate		0.55 ml
0.2M NaOH		700.00 ml
[7]Trace element solution		
$MnCl_2 \cdot 4H_2O$		0.250 g
$NiCl_2 \cdot 6H_2O$		0.250 g
H_3BO_3		0.250 g
$NaMoO_4 \cdot 2H_2O$		0.250 g
$FeSO_4 \cdot 7H_2O$		0.200 g
$NaVO_3 \cdot 4H_2O$		0.050 g
$CoCl_2 \cdot 6H_2O$		0.050 g
SeO_2		0.050 g
$ZnCl_2$		0.250 g
$CuCl_2 \cdot 2H_2O$		0.025 g

[8]Haemin solution		
Haemin		0.1g
Ethanol		10 ml
0.05M NaOH	Make up to 1L	~990 ml

Appendix Table 3. The components of buffer medium C, by Davies, (1991) adapted from Orpin, (1976) used to form the culture inoculum for the *in-vitro* experiments.

Buffer medium C components	In 1 L
Salts solution 1[1]	150 ml
Salts solution 2[2]	150 ml
Clarified rumen fluid	150 ml
Yeast extract solution (50 g L^{-1})	50.0 ml
Trypticase Peptone	10.0 g
$NaHCO_3$	6.00 g
Resazurin solution (0.1 % w/v)	1.00 ml
L-cysteine hydrochloride	1.00 g
Distilled water	~ 499 ml
[1]Salts solution 1	
K_2HPO_4	3.00 g
[2]Salts solution 2	
KH_2PO_4	3.00 g
$(NH_4)_2SO_4$	6.00 g
NaCl	6.00 g
$MgSO_4 \cdot 7H_2O$	0.60 g
$CaCl_2 \cdot 2H_2O$	0.60 g

Appendix Table 4. References used in the calculation of concentrations for the medium, high and extreme levels of inclusion, for the *in-vitro* experiments.

Mycotoxin	Level of inclusion	Dose µg/g	Reference
MPA[1]	Medium	0.222	Survey[5] (median; (Chapter 3)
	High	~5.00	Cow study (Chapter 5)
	Extreme	n/a	no information available[6]
FUS[2]	Medium	0.026	Survey (median; Chapter 3)
	High	n/a	no information available
	Extreme	15.0	May et al., 2000
DON[3]	Medium	~1.74	Dänicke et al., 2017; Winkler et al., 2014
	High	~4.95	Dänicke et al., 2017; Kinoshita et al., 2018; Winkler et al., 2014
	Extreme	8.05	Dänicke et al., 2005, FDA[7]
PEN[4]	Medium	0.746	Survey (median; Chapter 3)
	High	1.54	Survey (mean; Chapter 3)
	Extreme	10.5	Survey (maximum; Chapter 3)

[1]MPA: mycophenolic acid; [2]FUS: fusaric acid; [3]DON: deoxynivalenol; [4]PEN: penicillic acid;

[5]Survey: results from the Survey carried out on UK grass silages from 2022 – 2023;

[6]No information available in the literature or survey relevant to the particular level of inclusion.

[7]The United States Food and Drug Authority (2018)

Appendix Table 5. Mycotoxin content of silage used to “spike” the Spoiled silage clamp, that was then fed dairy cows over a five-day mycotoxin challenge phase.

Silage mycotoxin load	(µg/kg)	s.d[1]
Fusaric acid	509	112
Fumonisin B_2	30.2	1.54
Penicillic acid	1803	115
Cyclopiazonic acid	77.6	18.7

[1]s.d. = standard deviation. Only data for mycotoxins present above the level of detection are included.

Appendix Table 6. Universal prokaryotic primers used to target the V4 variable region of the 16S rRNA gene, as outlined by Kozich et al. (2013) with their prospective i5 and i7 sequence.

Forward primer	i5 sequence	Reverse primer	i7 sequence
SA501	ATCGTACG	SA701	AACTCTCG
SA502	ACTATCTG	SA702	ACTATGTC
SA503	TAGCGAGT	SA703	AGTAGCGT
SA504	CTGCGTGT	SA704	CAGTGAGT
SA505	TCATCGAG	SA705	CGTACTCA
SA506	CGTGAGTG	SA706	CTACGCAG
SA507	GGATATCT	SA707	GGAGACTA
SA508	GACACCGT	SA708	GTCGCTCG
SB501	CTACTATA	SA709	GTCGTAGT
SB502	CGTTACTA	SA710	TAGCAGAC