MANAGEMENT PRACTICES AND QUALITY OF FIRST-CUT ALFALFA-GRASS SILAGE ON CANADIAN DAIRY FARMS

by

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ABSTRACT

Fungal contamination of forages can cause decreased nutritive value, mycotoxin production and animal health issues. Ninety-three first-cut alfalfa-grass silage samples were collected from various storage types (wrapped round bales, conventional tower silos, horizontal bunker silos) on Canadian dairy farms. Quality analyses (near infrared reflectance [NIR], wet chemistry, digestibility analyses) and metagenomic sequencing were performed and effects of management factors on these parameters were evaluated. *Pichia* spp. yeasts capable of aerobic deterioration and mycotoxin-associated moulds of the genera *Monascus* and *Pencillium* were most abundant. Mycotoxin contamination was low with zearalenone (ZEA) present in 1% and mycophenolic acid (MPA) in 11% of samples. Higher yeast abundance in tower silos indicate they are not airtight and do not preserve quality well. Bales had lower heat damaged protein (ADF-CP) than other storage types despite a less complete fermentation. Findings will contribute to developing management plans to assist dairy producers in producing high quality silage.

LIST OF ABBREVIATIONS AND SYMBOLS USED

AAFC – Agriculture and Agri-Food Canada
ADF – acid detergent fiber
ADF-CP – acid detergent fiber crude protein
ANCOM – analysis of composition of microbiomes
ANOVA – analysis of variance
ASV – amplicon sequence variant
BF – by-sample frequency
cm – centimeter
CP – crude protein
DM – dry matter
DNA – deoxyribonucleic acid
DON – deoxynivalenol
E – expect value
ELISA – enzyme-linked immunosorbent assay
g – gram
h – hour
ITS – internal transcribed spacer
LAB – lactic acid bacteria
LC-MS/MS – liquid chromatography tandem mass spectrometry
m – meter
min – minute
mL – milliliter
mm – millimeter
mM – millimolar
MPA – mycophenolic acid
NDF – neutral detergent fiber
NFC – nonfibrous carbohydrates
NGS – next generation sequencing
NIR – near infrared reflectance
PCR – polymerase chain reaction
QIIME – quantitative insights into microbial ecology
RA – relative abundance
rcf – relative centrifugal force
s – second
S.D. – standard deviation
SS – similarity score
TDN – total digestible nutrients
TF – total frequency
UQAT – Université du Québec en Abitibi-Témiscamingue
WC – wet chemistry
ZEA – zearalenone
μg – microgram
μL – microliter
μm – micrometers

μM – micromolar
°C – degrees Celsius

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Chapter 1: Introduction

1.1 Introduction

Canadian dairy producers are under pressure to remain economically viable while facing issues such as low milk prices, high input costs, and the threat of other countries gaining more access to the Canadian market. Reducing the cost of production enables producers to be more environmentally and economically sustainable and compete domestically and internationally. There are 10,525 dairy farms in Canada (Statistics Canada 2016a), milking a combined 969,700 cows (Statistics Canada 2018). Forages make up between 40-90% of the feed requirements of ruminants (Charmley 2001) and conditions in Canada are favourable for growing cool season grass and legume forage crops due to its temperate climate (Bernardes et al. 2018). Land dedicated to alfalfa and alfalfa-based mixtures makes up 3,754,169 ha, while tame hay and fodder crops constitute 1,963,197 ha (Statistics Canada 2016b). Forage is commonly conserved as silage because it requires less field wilting than hay. This process involves lactic acid fermentation causing a rapid lowering of pH in anaerobic conditions, preserving forage. The most commonly ensiled forages in temperate areas are alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.) and grasses including corn (*Zea mays* L.) (Kalač 2011). Forage can be harvested by animals in pastures, or mechanically harvested as silage, haylage, or hay. Since animals cannot be pastured year-round in Canada, and hay requires a longer wilting period in the field, silage is an attractive option and makes up a large component of feed on Canadian dairy farms. Silage production is commonly used to preserve perennial forages on dairy farms, allowing for easier feeding than hay because it

is more easily incorporated into total mixed rations and in mechanized feeding systems (Mahanna and Chase 2003).

The production of silage from on-farm perennial forages is an economical and environmentally friendly way to reduce dependence on purchased feed as the production and processing of purchased feed components results in 2 to 5 times more greenhouse gas emissions than forage crops (Adom et al. 2012). Feed is the single highest expense for many Canadian dairy farms (Van Biert 2017) and its cost can be minimized by growing on-farm perennial forages because they do not have to be re-established every year (un-like annual crops) which lowers expenses (Ojeda et al. 2018). Alfalfa is the most widely grown perennial legume and can be grown alone or with temperate and tropical grasses (Capstaff and Miller 2018). Growing perennial forage such as alfalfa can be advantageous in farming systems because of its ability to sequester carbon and to reduce erosion, greenhouse gas emissions, nitrate leaching, reliance on fertilizer, and costs of production in comparison to annual crops (Neal et al. 2013). Perennial forages are a low-cost feed source that can be used to lower costs of production, but they need to be high quality for animals to be healthy and productive (Capstaff and Miller 2018). Producing consistently nutritious and palatable silage to feed productive and healthy cows is challenging because of the many factors that affect silage quality.

Some climatic conditions which are favourable for cool season perennial forage production are also ideal for fungal growth; moist conditions promote fungal growth, especially since silage is conserved at a higher moisture content than hay (Pan et al. 2009). Many regions of Canada have a cool humid climate which is well suited for forage production, but this humidity is also ideal for the growth of spoilage and silage

deterioration (Bernardes et al. 2018). Silage quality can be evaluated by determining the presence of fungal contaminants which may produce mycotoxins, a diverse group of small molecular weight compounds produced by fungi which can result in disease and death in humans and animals (Bennett and Klich 2003).

Fungal contaminants have been found in Newfoundland (Jewell, personal communication) but the risk of mycotoxin exposure is not clear across Canada; there is thus a need to evaluate the effects of silage practices on quality across the range of conditions found on Canadian dairy farms. Silage has been the focus of a great deal of research. However, most of the research does not deal with silage that is being fed to dairy cattle on working farms. Canadian dairy producers are under pressure to become more competitive; they require knowledge from research conducted in Canada to assist them in making decisions regarding silage production and management.

The main goal of this project is to assess the presence of organisms that could produce mycotoxins in silage on Canadian dairy farms and evaluate relationships between management practices and silage quality parameters.

1.2 Literature Review

The spoilage of silage with fungi may result in severe consequences for animal health and the bottom line of dairy producers. There has been a great deal of research done on fungal contaminants in cereal grain crops, but little research has focused on fungal contamination of silage, especially in Canada. The contamination of silage with fungal contaminants decreases the nutritive value of the silage (McDonald et al. 1991) and can cause mycotoxin production which can have ramifications for animal health and performance (Scudamore and Livesey 1998). Fungal contaminants from the genera

Fusarium, *Aspergillus*, and *Penicillium* are particularly concerning as they include mycotoxin associated fungi that have been found to present risks to animal health. Microfungi display mould- or yeast-type growth forms. While both yeasts and moulds are fungi, yeasts are single-celled organisms that reproduce by budding, and moulds produce long multicellular filaments called hyphae (McGinnis and Tyring 1996).

1.2.1 High Feed Costs on Dairy Farms

A study done in 2015 reported that feed costs were 52.3% of total operating costs on Alberta dairy farms (Van Biert 2017). Some producers may be inclined to lower feed costs in an attempt to reduce operating costs (VandeHaar and St-Pierre 2006). It is important that producers provide feed that is cost-effective while not sacrificing the proper nutrition that is necessary for high milk production, as allowing cows to achieve their genetic potential for milk production is usually more profitable than lowering feed costs (VandeHaar and St-Pierre 2006). A 2013 Cornell Dairy Farm Business Summary revealed that farms in the top 20% for return on their assets differed from other farms in terms of feed; in comparison to the average farm, the top 20% of farms actually spent 3.1% less on purchased feed and 3.7% less on feed grown on farm, while maintaining higher milk production (Karszes et al. 2013). These farms reduced feed costs and increased milk production with high forage quality (Karszes et al. 2013). The Dairy Cost Study in Alberta had similar findings; this study reported that in 2015 the bottom one third of Alberta dairy farms (i.e. those with the highest cost of milk production) paid $4.28 more per hectoliter for feed costs in comparison to the top one third of farms and also had lower milk production (Van Biert 2017).

High quality forage is key in reducing feed costs, increasing milk production and, therefore, remaining profitable. When best management practices for silage making are

followed, high quality feed for ruminants is achieved (McDonald et al. 1991). The desired end product of ensiling forage is a feed with high energy, high recovery of dry matter, and highly digestible nutrients that is free of undesirable compounds that could impede animal performance (Kung et al. 2018). Evaluating the quality of silage can be achieved by measuring the pH and quantifying the production of organic acids and mycotoxins (Kung et al. 2018).

1.2.2 Silage

Ensiling is the preservation of moist forage crops in a confined structure (silo) in the absence of oxygen to retain nutrient content. Ideal silage preservation involves lactic acid fermentation converting water-soluble carbohydrates into lactic acid, causing a rapid lowering of pH in anaerobic conditions. Under these anaerobic conditions, anaerobic and facultative microorganisms multiply and ferment sugars and organic acids in the forage. The most common fermentative microbial groups are lactic acid bacteria (LAB), *Clostridia* spp., and yeasts, with lactic acid being the most desirable (Pahlow et al. 2003). After forage is sealed in a silo, LAB, enterobacteria, *Clostridia* spp. and yeasts compete for nutrients. The first few days following ensiling determines the fermentation process that takes place; in ideal conditions, LAB will rapidly create an acidic environment unsuitable to other organisms which will create a stable silage with a low pH (McDonald et al. 1991). In the event that the pH is not lowered quickly, unwanted microorganisms such as *Clostridia* spp., enterobacteria and yeasts will compete with LAB for nutrients which will likely result in an unstable silage because the products that these undesirable microorganisms produce do not contribute to preservation of the silage and may negatively affect its nutritive value (McDonald et al. 1991). Fermentation by enterobacteria and *Clostridia* spp. is not desirable and is likely to occur if the forage is

low in LAB and water-soluble carbohydrates, has a high buffering capacity, or is too wet (McDonald et al. 1991).

Forages with high buffering capacities are more resistant to a change in pH. Legumes such as alfalfa have higher buffering capacities and low fermentable sugars in comparison to other forages, making them difficult to ensile (McDonald et al. 1991). The high buffering capacity of alfalfa requires more acid to lower the pH for a successful fermentation. An adequate amount of LAB must be produced to inhibit growth of *Clostridium* spp.; this is usually achieved at a pH of 4.2 (McDonald et al. 1991). Legume silages can have a pH higher than 4.6 to 4.8 if ensiled too wet at less than 30% dry matter (DM) and may undergo clostridial fermentation which leads to poor quality silage (Kung and Shaver 2001). Silage may also have a higher pH if the forage is too dry (more than 45-50% DM) and as a result, fermentation is hindered. This makes the silage more unstable when exposed to oxygen since not enough acid is produced to discourage secondary microbial growth (Kung 2010). Clostridial fermentation can cause protein breakdown and the production of ammonia in the silo as well as the accumulation of butyric acid which results in lower quality forage (McDonald et al. 1991; Pahlow et al. 2003; Kung 2010). It is important that silage is stored at the correct DM and packed tightly to reduce initial oxygen and to reduce ammonia concentration (Kung 2010).

LAB convert water-soluble carbohydrates into lactic acid, which preserves the forage and hinders the proliferation of spoilage microorganisms (Filya et al. 2000). Maintaining anaerobic conditions is very important for lactic acid to dominate the fermentation (McDonald et al. 1991). Under ideal conditions, growth of moulds that break down sugars and lactic acid are inhibited but may appear on the sides and surface

of silage where there is oxygen exposure (McDonald et al. 1991). Silage can be exposed to oxygen by damage to the silo or during the feed out phase when the silo is opened to remove feed for animals. This exposure to oxygen can cause spoilage microorganisms present to multiply. The growth of yeasts and moulds results in nutrient losses and aerobic deterioration of silage DM (Pahlow et al. 2003). The amount of loss is dependent on storage type and management (Wilkinson and Davies 2013).

Silage quality is affected by a broad range of parameters including temperature (Koc et al. 2009), oxygen content (Muck and Huhnke 1995; Pahlow et al. 2003), length of chopped forages (Ruppel et al. 1995; Kung et al. 2018), moisture content (Coblentz and Atkins 2019), percent legume in the forage stand (Xue et al. 2020), maturity at harvest (Cassida et al. 2000), and use of inoculants (Kung et al. 2018). The type of silo (McElhinney et al. 2015), density of forage in the silo (Ruppel et al. 1995; Han et al. 2004; Kung et al. 2018), and feed out management also play a role in silage quality (Muck and Huhnke 1995; Ruppel et al. 1995; Kung et al. 2018).

1.2.3 Silage Storage Systems

In Canada, silage is commonly stored in upright tower silos, horizontal bunker silos, or as wrapped bales, where silage is baled and wrapped with polyethylene film plastic to exclude oxygen. Silage can be chopped into smaller pieces during the harvesting process in order to achieve higher quality forage and a more successful fermentation with higher lactic acid levels and lower butyric acid levels (Murdoch et al. 1955). Substrate availability for LAB is influenced by chopping of the forage. LAB populations increase after chopping silage because this process allows plant sugars to diffuse outside of the plant and to be readily available for LAB growth (Muck 1989). The forage used to make baled silage is not necessarily chopped which results in bales having

a high surface-to-volume ratio and making it susceptible to aerobic deterioration (Weinberg and Ashbell 2003).

Baled silage is preserved at a higher DM concentration and stored at a lower bulk density in comparison to other conventional silages. It also has a thinner plastic barrier and has 6 to 8 times the surface area in contact with the plastic film compared to bunker silos (O'Kiely et al. 2002). Approximately half of the silage volume is within 15 centimeters (cm) of the plastic film which could leave it susceptible to air penetration and mould growth (O'Kiely et al. 2002). The plastic wrap that is used to conserve silage can be damaged (e.g. by animals or equipment) which can introduce oxygen into the silage holes, allowing fungi and other organisms to grow (McNamara et al. 2001). The plastic film, bale density, and forage DM concentration all play a role in silage quality (O'Kiely et al. 2002). Han et al. (2004) found increasing silage bale density of alfalfa silage by packing the bales more tightly to result in improved silage quality with increased lactic acid concentrations with lower pH. O'Brien et al. (2008) were the first to conduct an extensive survey of moulds and yeasts on baled silage in Ireland. Visible fungal contamination was common on the 180 farms involved with the survey.

Silage that is stored in tower silos and horizontal bunkers is chopped. Forage can also be stored in tower silos, which are tall cylindrical structures that use gravity to pack forage and may be the most ideal way to store forage because there is limited surface exposure to the air (McDonald et al. 1991). The weight of the forage material on the top of the silo compresses the material below, resulting in decreasing density from the top of the silo to the bottom. Horizontal bunkers are usually filled with silage in progressive wedges and packed with tractors driving back and forth on the forage as soon as possible

after storage to compact it and remove oxygen, and thus reduce losses from plant respiration and aerobic microorganisms (Weinberg and Ashbell 2003). Bunker silos are then covered with plastic as quickly as possible to create an anaerobic environment and encourage good fermentation (McDonald et al. 1991).

Research on silage conducted on the Avalon Peninsula of Newfoundland in 2016 observed that fungal contamination was higher in samples taken from bunker silos than samples taken from wrapped bales, with members of the genera *Penicillium* and *Aspergillus* detected in cultures taken from both bunkers and bales (Jewell, personal communication). These findings could be because of differences in DM between the storage systems; if silage has a low DM, fungi such as *Penicillium* and *Aspergillus* can grow which elicits a rise in temperature which can encourage other fungi to grow, further degrading the silage (Alonso et al. 2013). However, it is more likely that the silage in the bunkers had higher aerobic deterioration due to improper storage techniques such as an uneven face when feeding to animals and improper sealing of the plastic (Jewell, personal communication). Research conducted at the University of Vermont found similar results when evaluating mycotoxin contamination in various storage systems. Horizontal bunker silos that were left open to oxygen had the highest concentration of toxins (Gotlieb 1999). High levels of mycotoxins were related to poor management of the silo and ingress of oxygen into the silage, whereas there was no significant difference in levels of toxin in well managed bunkers that were covered with plastic and weighted with tires in comparison to well managed upright silos. Gotlieb (1999) suggests that oxygen is the deciding factor in the development of toxins in silage since temperature and moisture are sufficient for toxin production. Silos that use plastic coverings slow oxygen ingress but

do not eliminate it which can allow eventual fungus growth and toxin contamination (Gotlieb 1999).

While silage in horizontal bunkers and tower silos is chopped, baled silage may not be. Nicholson et al. (1991) compared baled silage with bag silage (ag bags), which is a tube of plastic filled with chopped forage and most comparable to a bunker silo. It was found that LAB in the bag silage multiplied quickly, causing a more desirable fermentation than the baled silage, most likely because the baled silage was not chopped, and lower levels of plant sap were available for LAB as substrate. The bag silage was more stable and had lower *Clostridia* counts, higher DM intakes, and faster animal gains (Nicholson et al. 1991).

Due to the fact that the long plant stems in round bale silage do not release plant sugars as quickly as chopped silage, fermentation may be restricted. Round bale silage is normally drier than chopped silage which also hinders fermentation as higher forage moisture results in increased fermentation acids produced (Lindquist 2017). Bales typically have a lower density than forage stored in bunker silos, which means there is more oxygen in the bales that can result in delayed fermentation, causing more sugars to be metabolized by plant cells (Bernardes et al. 2018). Lactic acid populations are normally effective in preventing mould growth but even a small amount of oxygen can cause fungi such as *Penicillium roqueforti* and *Penicillium paneum* to grow (Gallo et al. 2015). LAB populations are lower in bales which could result in failure to achieve a lower pH and perhaps a higher rate of development of fungi.

1.2.4 Mycotoxins

Mycotoxins are secondary metabolites of moulds associated with several genera, in particular *Aspergillus*, *Fusarium*, and *Penicillium* spp., affecting a variety of crops

including cereals, forage legumes and grasses, which can cause harmful responses in humans and animals (Scudamore and Livesey 1998; Fink-Gremmels 2008; Zain 2011; CAST 2013). Moulds are filamentous fungi which may produce mycotoxins depending on a variety of factors including pH, oxygen, temperature, and moisture. Filamentous fungi have been observed on silage, but the appearance of mould is not always indicative of mycotoxins (Diaz-Nazario 2002). If the conditions are conducive for moulds to grow, then mycotoxin production is possible; on the other hand, mycotoxins may still be present even though mould is not visible to the naked eye (Diaz-Nazario 2002).

The presence of mycotoxins in silage may occur because of infection of the crop in the field (Driehuis 2013). Microbial contaminants such as bacteria, yeasts and moulds are present in the field and naturally come into contact with forage used for silage through air, insects, and rain splash. Yeasts and moulds that could produce mycotoxins may also be introduced during harvesting, transport and storage (Magan and Aldred 2007). The bacteria and fungi present on plants are normally concentrated on the lower leaves and stems where they are less at risk of ultraviolet radiation and drying (Blakeman 1981), but *Fusarium*, *Aspergillus*, and *Penicillium* can also be found in soil and the air (CAST 2003; Pahlow et al. 2003). *Clostridia* are bacteria which can be present in soil, manure, and on forage (Pahlow et al. 2003).

Forage is susceptible to a range of fungi which may infect crops and produce mycotoxins if the conditions are appropriate. Cool, damp weather and drought conditions which cause plants to become stressed can encourage mycotoxin development (Scudamore and Livesey 1998). Further mould growth and mycotoxin development in storage from field fungi is unlikely if silage is stored properly and anaerobic conditions

are achieved. Should oxygen gain access to the silage, storage moulds such as *Penicillium* and *Aspergillus* may develop with species development dependent on physical presence, temperature and water activity (Scudamore and Livesey 1998). Some of the organisms that have been isolated from silages in temperate climates include filamentous fungi, such as *Penicillium roqueforti, P. paneum*, *Schizophyllum commune* (O'Brien et al. 2008), *Aspergillus fumigatus* (Richard et al. 2009), as well as yeasts including *Pichia fermentans*, *Pichia anomala*, and *Geotrichum* spp. (O'Brien et al. 2008).

The presence of mycotoxins in livestock feed can have detrimental effects on animal health and is a growing concern. Forage crops were found to cause occasional complications in animal health, especially in temperate climates (Scudamore and Livesey 1998) and mycotoxins, even at low doses, can negatively affect the immune system (Fink-Gremmels 2008). The consumption of feed materials contaminated with mycotoxins by dairy cows can result in feed refusal, reductions in feed quality, palatability, and intake, as well as poor weight gain, bleeding, birth defects and kidney, liver, or lung damage, subclinical losses in milk production, reproduction issues, increases in disease incidence, and even death in extreme situations resulting in serious economic losses (Scudamore and Livesey 1998; Diaz-Nazario 2002). The consumption of mycotoxins usually results in chronic conditions which suggest that there are other hidden health effects; if clinical disease issues are arising, it is predicted that sub-clinical exposure is occurring more frequently (Scudamore and Livesey 1998). Identifying mycotoxins as the cause of diseases and other issues can be challenging because of nonspecific symptoms and difficulties associated with feed sampling and analysis, and

the presence of other stresses. When animal productivity problems arise, mycotoxin contamination should be contemplated as a possible factor (Diaz-Nazario 2002).

According to CAST (2003), the major classes of mycotoxins include aflatoxins, trichothecenes, fumonisins, zearalenone, and ochratoxin. These mycotoxins present the greatest risk to human and animal health due to their contamination of food products, but other mycotoxins such as sterigmatocystin and mycophenolic acid occur as well (CAST 2003).

Aflatoxins are produced primarily by *Aspergillus flavus* and *A. parasiticus* (CAST 2003; JECFA 2001). The major aflatoxins are B1, B2, G1, and G2, with *A. flavus* responsible for the production of B aflatoxins and *A. parasiticus* for both B and G aflatoxins (JECFA 2001). Aflatoxins are considered the most potent animal carcinogen (Squire 1981).

Fumonisins are a class of mycotoxins produced by *Fusarium* spp., primarily *F. verticillioides* and *F. proliferatum*, common pathogens of corn (JEFCA 2001). Fumonisin B1, regarded as the most toxic fumonisin, has been found to cause leukoencephalomalacia in horses (Kellerman et al. 1990), and hepatocarcinoma in rats (Voss et al. 1990). Caloni et al. (2000) reported Fumonisin B1 to be poorly metabolized in the rumen in ruminants and underwent limited degradation.

Several fungal genera produce trichothecenes, especially species belonging to the genus *Fusarium* (CAST 2003). The most frequent trichothecene is deoxynivalenol (DON), a common cereal contaminant (CAST 2003), produced by *F. graminearum* and *F. culmorum* (JECFA 2001). Contamination of feed with DON can initiate acute gastroenteritis causing vomiting as well as reduced feed intake and growth, with pigs

being highly susceptible, while poultry and ruminants are comparatively resistant (Pestka 2007). 3- and 15- acetyl DON can also contaminate cereals but are equivalently or less toxic than DON (Pestka 2007).

Zearalenone (ZEA) is a metabolite produced by some *Fusarium* species such as *F. graminearum* and is associated with strong estrogenic activity, and so it is also referred to as a phytoestrogen and a mycoestrogen (Bennett and Klich 2003). ZEA contamination in feed has been linked to reduced conception rates (Weaver et al. 1986) and early abortions (Kallela and Ettala 1984).

T-2 and HT-2 toxins are type-A trichothecene mycotoxins, produced mainly by *F. sporotrichioides*, and occasionally by *F. poae, F. equiseti,* and *F. acuminatum* (JECFA 2001). T-2 toxin is metabolized to HT-2 toxin after ingestion (Kiessling et al. 1984). T-2 is a potent mycotoxin found to initiate multiple adverse acute and chronic health effects in ruminants (Ferreras et al. 2013).

Diacetoxyscirpenol is another type-A trichothecene produced by several *Fusarium* species including *F. langsethiae, F. poae, F. sporotrichioides* and *F. sambucinum* (Knutsen et al. 2018), that is less cytotoxic to humans and rats than T-2 and H-T toxins, but more toxic than DON (Lautraite et al. 1997). It has been reported to cause haematological issues in rats and humans (Lautraite et al. 1997).

Ochratoxin A is produced by fungal species such as *A. ochraceus*, *A. carbonarius*, *A. niger* and *Pencicillium verrucosum* (JEFCA 2001). This mycotoxin has been reported to impair immune function (Lea et al. 1989). Kiessling et al. (1984) reported zearalenone, T-2 toxin, diacetoxyscirpenol and deoxynivalenol to be well-metabolized by whole rumen fluid, but aflatoxin B1 and ochratoxin A were not. Ruminants are not as

susceptible to ochratoxin A as non-ruminants, and it was believed that ochratoxin A was completed degraded by the rumen of ruminants and adverse health effects were not evident. However, Mobashar et al. (2010) reported ochratoxin A to be a greater risk than previously understood and that the rumen cannot completely degrade this mycotoxin.

The mycotoxin sterigmatocystin is produced by many filamentous fungi, notably *A. versicolor* and *A*. *nidulans* and is a precursor of aflatoxin biosynthesis (Barnes et al. 1994). Mycophenolic acid, produced by *Penicillium* species, especially *P*. *roqueforti*, is an antibacterial, antifungal, antiviral mycotoxin (Abraham 1945; Abrams and Bently 1959; Cline et al. 1969) that also has low antimicrobial activity and immunosuppressive effects (Bentley 2000). Information on the effects of MPA on ruminant health are limited.

Mycotoxins are commonly detected using two methods, enzyme-linked immunosorbent assay (ELISA) and liquid chromatography tandem mass spectrometry (LC-MS/MS). ELISA is an immunochemical method that provides rapid screening for mycotoxins but analytical methods such as high-performance liquid chromatography are superior, particularly when used with tandem mass spectrometry as the determination of multiclass mycotoxins can be achieved in a single analysis.

1.3 Research Gap and Objectives

1.3.1 Research Gap

There is a lack of research done on the contamination of forage crops with mycotoxins and the potential issues that this may cause (Scudamore and Livesey 1998; Alonso et al. 2013). Gallo et al. (2015) points out that the number of published articles on research related to mycotoxin contamination in forage is very small in comparison to the articles published on mycotoxin presence in cereals. Although there is an increasing

understanding of mycotoxin occurrences in silage, data are still limited in this area of research. Because of this lack of research, the risk of mycotoxin exposure in ruminant species cannot be properly assessed. Gallo et al. (2015) strongly recommend that forage be tested not only for nutritive and fermentative characteristics, but also mycotoxin contaminations. Scudamore and Livesey (1998) echo a similar message; they believe that future research should focus on identification of mycotoxins, especially those which introduce residues into meat or animal products, even if these mycotoxins are only present at sub-clinical levels in animals. Determining if mycotoxin contamination is occurring is important because the use of silage makes up such a large proportion of a dairy cow's diet (Charmley 2001) and the potential threats to human and animal safety must be assessed (Scudamore and Livesey 1998).

The mycotoxin-producing species found in silage from dairy farms in Newfoundland warrants further research on mycotoxin detection to assess possible impacts on the dairy industry. There are many reasons why a more detailed study is needed to detect the presence of fungal contamination in silage: the high level of microorganisms that were observed in Newfoundland, the comments and concerns of producers, and the research that has been documented by others in similar climates (especially Ireland) (Jewell, unpublished).

1.3.2 Research Objectives

The objectives of this project are to test silage samples for presence of mycotoxin-associated fungal species with the goal to identify relationships between silage management and quality parameters. Research findings will be used to develop management plans that are specific to different types of silage storage systems in different regions of Canada in order to reduce risks to the cost of milk production,

minimize negative environmental impacts and improve cow health and longevity. The following objectives have been designed to achieve this:

1) To detect and identify fungal species and populations in silage, and to determine whether mycotoxin-associated species are present in silage

2) To evaluate the relationships between silage management and quality parameters identified using factor analysis.

This research contributes to a larger project titled “Identification of best management practices for high quality silage production tailored to different silage systems and regions across Canada through survey and analyses of economics, nutrition, and microbial contamination”.

This thesis presents information on the detection and identification of fungal species and populations in silage in Chapter 2. Chapter 3 evaluates the relationships between silage management and quality parameters. General conclusions and future research are presented in Chapter 4.

Chapter 2: Detection and Identification of Fungal Species and Populations in Silage

2.1 Introduction

Yeasts and moulds are regarded as being the primary contributors to silage aerobic deterioration (Wilkinson and Davies 2013). Yeasts are single-celled fungi that reproduce by budding, while moulds are fungi that produce long multicellular filaments called hyphae (McGinnis and Tyring 1996). Some fungi can grow as either moulds or yeast cells and are referred to as dimorphic. The growth style of dimorphic fungi is affected by factors such as temperature and pH; for example, some dimorphic fungi grow as yeasts at 37°C, but as moulds at 25°C (McGinnis and Tyring 1996).

The growth of moulds is slow in comparison to other microorganisms in silage (Muck 2010). Seldom are moulds present at populations that will affect silage quality unless the silage has had significant aerobic deterioration by yeasts (Muck 2010). The visual appearance of filamentous fungi (moulds) is evidence that silage has lost a great deal of quality, but the greater concern is potential mycotoxin production from moulds. Mycotoxins are secondary metabolites produced by filamentous fungi that are capable of causing disease and death in humans and other animals, with many mycotoxins having overlapping toxicities to invertebrates, plants and microorganisms (Bennett 1987; Bennett and Klich 2003). Secondary metabolites are low-molecular mass molecules that are not directly necessary for growth and development of the organisms that produce them, unlike primary metabolites (Brakhage 2013). Production of mycotoxins occurs when moulds are under stress, with specific environmental stressors that cause this response differing across species (Muck 2010). Although mycotoxins do not pose serious issues to

the preservation of ensiled forage, they are of concern when considering the health effects on animals that consume mycotoxin-associated silage (Muck 2010).

Despite the possible ramifications for animal health due to mycotoxin-associated fungi, there has been little research conducted on detecting and identifying fungal species and populations in silage, especially in Canada. Kelman (2016) investigated mycotoxins in Canadian silage and proposed that this information would provide a risk assessment for the possible mycotoxins present in cooler climates, as climate change may influence the geographic distributions of mycotoxigenic fungi (Russell 2010). This means that mycotoxins normally detected in warmer climates may become present in Canadian crops (Kelman 2016). While this study answered many questions about silage in Canada, in regard to mycotoxin contamination in grass, barley and corn silage, there were no samples collected from alfalfa silage. Alfalfa silage is a large component of feed on Canadian dairy farms and is difficult to ensile in comparison to other silages (McDonald et al. 1991), which may result in different mycotoxins being present.

2.1.1 Internal Transcribed Spacer Region

Novel technology allows the detection and identification of fungal species at an efficiency not seen before. Fungal barcoding involves sequencing short sections of deoxyribonucleic acid (DNA) to identify fungal species (Mahmoud and Zaher 2015). The region of fungal DNA that is barcoded must be present in the majority of fungi but have sufficient diversity in order to distinguish between different species (Mahmoud and Zaher 2015). Schoch et al. (2012) proposed that the internal transcribed spacer (ITS) region be used as the primary fungal barcode marker as research conducted across a broad range of

fungi found the ITS region to have the highest probability of successful identification in the regions of the ribosomal gene (Schoch et al. 2012).

2.1.2 Next-Generation Sequencing

DNA sequencing information has traditionally been obtained using Sanger sequencing developed by Sanger et al. (1977). This sequencing technology provided a wealth of information but is limited due to its low throughput (the amount of DNA that can be processed at once) which resulted in newer sequencing technologies being developed (Churko et al. 2013). Next-generation sequencing (NGS) is used to sequence millions of small fragments of DNA in parallel which are then pieced together using bioinformatics analyses (Behjati and Tarpey 2013). These technologies are used to sequence entire genomes or certain areas of interest such as individual genes and is a more efficient and cheaper approach than the previous Sanger sequencing technology (Kircher and Kelso 2010; Behjati and Tarpey 2013). Bioinformatic tools are used to analyze the extensive amount of raw DNA sequence data produced to better understand microbial communities. NGS allows the study of fungal diversity in a more cost-effective way through producing large numbers of sequences from various environmental samples in addition to in-depth analyses (Yang et al. 2018). One of the most popular NGS technologies is Illumina, which can produce millions of short (200-500 bp, depending on the instrument) sequences from a single sample (Slatko et al. 2018).

2.1.3 Metagenomics

Metagenomics is the analysis of DNA from microbial communities in environmental samples (Handelsman et al. 1998). Microbial ecology studies have benefitted from the advanced technology of NGS with the field of metagenomics (Oulas

et al. 2015). The community and taxonomic distribution of environmental samples can be studied rapidly with polymerase chain reaction (PCR) amplification and sequencing of conserved marker genes (Oulas et al. 2015). The ideal genes are conserved enough for relatively generic primers to attach and synthesize DNA, but diverse enough to allow for differences to be observed between taxonomic groups. Associated environmental data (metadata) can be compared to the taxonomic distribution of the community (Oulas et al. 2015). NGS produces valuable information on the taxonomy of silage microbiota and is expected to become more widespread (Ni et al. 2017). Previous research on the fungal microbiota of silage relied on culturing for identification (O'Brien et al. 2008) whereas NGS is culture-independent technology (Duniere et al. 2017). Since many species cannot be cultured, microbial communities described by culturing methods underestimate microbial diversity (Duniere et al. 2017). Both culturing and NGS methods have been reported to have some taxonomic biases (Johnston et al. 2017). Romão et al. (2017) and Anguita-Maeso et al. (2020) suggest both approaches be used in parallel to provide more information as they offer complementary insights.

Computational tools are necessary to analyze the data produced by metagenomics in order to accurately investigate the community being studied (Lindgreen et al. 2016). A number of sequencing pipelines have been developed to analyze and interpret communities using bioinformatic analyses (Lindgreen et al. 2016). Straub et al. (2019) compared the performance of the most commonly used sequencing analysis tools, Mothur (Schloss et al. 2009), QIIME1 (Caporaso et al. 2010), QIIME2 (Bolyen et al. 2019), and MEGAN (Mitra et al. 2011). QIIME2 was reported to outcompete the other tools in terms

of sequence recovery, taxonomic assignments, and diversity estimates (Straub et al. 2019).

The application of NGS technology in silage has mainly focused on better understanding bacterial communities in silage; Eikmeyer et al. (2013) were the first to use NGS to taxonomically describe the bacterial microbial communities in grass silage while Ni et al. (2017) compared the bacterial communities of Italian ryegrass with whole crop corn and alfalfa. Much of the research conducted on bacterial communities in silage using NGS focuses on inoculant studies such as Zheng et al. (2017). Other researchers have taken advantage of NGS technology to better understand fungal communities in silage; Duniere et al. (2017) and Romero et al. (2017) used NGS to describe fungal communities in small grain silages. Other researchers have used NGS to investigate both the fungal and bacterial microbial communities in silage such as the Canadian study by Kelman (2016) on contaminated corn, barley and grass silage and the study by Romero et al. (2018) on the evaluation of inoculants in corn silage. There appears to be a research gap in fungal communities in legume plus grass silage using NGS.

2.1.4 Research Objectives

The objectives of this project are to detect and identify fungal species and populations in silage, and to determine whether mycotoxin-associated species are present in silage.

2.1.5 Research Hypotheses

1) Fungi presence and abundance will differ across regions in Canada due to regional factors

2) Similar fungi will be detected as those found in silage in temperate climates by other researchers; some fungi may include *P. roqueforti, P. paneum*, *Schizophyllum commune* (O'Brien et al. 2008), *Aspergillus fumigatus* (Richard et al. 2009), and yeasts including *Pichia fermentans*, *Pichia anomala*, and *Geotrichum* spp. (O'Brien et al. 2008).

2.2 Materials and Methods

2.2.1 Silage Collection

Silage samples were collected from farms in three regions across Canada (Atlantic Canada, Central Canada (Ontario and Quebec), and Western Canada (Prairies and British Columbia)) for two years by graduate students, university faculty members, extension workers and industry professionals. Dalhousie University was responsible for collecting samples from New Brunswick, Nova Scotia and Prince Edward Island, while Agriculture and Agri-Food Canada (AAFC) St. John's sampled Newfoundland. Université du Québec en Abitibi-Témiscamingue (UQAT) collected samples from Quebec and Ontario. Samples from the Prairies were collected by the University of Manitoba in Manitoba and AAFC Agassiz in British Columbia.

There was a total of 93 silage samples collected from the 2018 harvest year with 30 samples collected from round bales, 30 from horizontal bunker silos, 28 from top-unloading tower silos, 3 from piles, and 2 from ag bags. There were 124 silage samples collected from the 2019 harvest year with 36 samples collected from round bales, 42 from horizontal bunker silos, 32 from top-unloading tower silos, 10 from piles, and 4 from ag bags. Samples collected ranged from having no alfalfa to high levels of alfalfa. Data presented in this chapter is from silage harvested in 2018 as data from 2019 was not yet available.

All samples from the Atlantic and Quebec and Ontario regions were collected in winter after harvest. Samples from the Prairies and British Columbia were collected in summer of the harvest season. All samples were from primary (first cut) forage. The intention was to collect a total of 750 g of silage with 150 g of silage collected to be sent to Dalhousie University in Truro, Nova Scotia for fungal and mycotoxin analysis, and 600 g collected to be sent to Ontario and Quebec and Western Canada for NIR (near infrared reflectance) and wet chemical analyses, but some samples were smaller. In bunker silos, the front-end loader of a tractor or a defacer was used to bring down a pile of silage and handfuls were collected, or handfuls of silage were removed from the face of the bunker in a "W" pattern. A quartering method was used to collect a representative sample for a final sample of 750 g. The silage collected in the bucket was divided into four sections and two opposite quarters were removed. The two remaining quarters were mixed in the bucket and a representative sample was taken. A similar method was used when collecting from tower silos, except handfuls of silage were taken from the conveyor belt as silage came out of the silo. A forage probe (Model number 160, Star Quality Samplers, Irricana, Canada) attached to a Milwaukee drill was used to collect samples from round bales in the Maritimes. A knife was used to make a slit in the plastic covering the bale and then the probe was inserted at least 10 times in at least one bale to ensure a representative sample. Holes made in bales were covered up using silage tape to ensure oxygen was not gaining entry to the bales after sampling. The quartering method was not used when sampling round bales; instead the cores were placed in a bag as is. Samples were placed in coolers filled with ice packs and transferred to a freezer as soon as possible so that DNA quality was not compromised.

2.2.2 DNA Extraction from Silage Samples

Whole genomic DNA was extracted from silage following the methods of Gallagher et al. (2018) with some modifications. Ten g of silage was weighed and placed in a sterile, DNA free 50 mL Falcon tube (Fisher Scientific) that was filled with 30 mL of phosphate buffered saline tablets (pH 7.4) (VWR) with each tablet preparing 1X PBS solution of 137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer when dissolved in 100 mL of water. Three tubes were filled for each sample for a total of 30 g of silage used. Samples were left to soak in the tubes for 30 min before vortexing for 45 s, inverting every 15 s. The supernatant was poured through a sterile cotton ball into a clean tube and samples were centrifuged at 9,223 rcf (relative centrifugal force) for 30 min at 10°C. The supernatant was discarded, and the pellets were resuspended in 1 mL of sterile water, then centrifuged at 10,000 rcf for 5 min and the supernatant discarded.

The pellet obtained was transferred to the DNeasy PowerSoil Kit (Model 12888-100, Qiagen) using sterile disposable spatulas (Fisher Scientific). The pellet was divided into two tubes of approximately 0.3 g each to avoid overloading the kit. The kit was used according to the manufacturer's instructions with the following modification: 50 µL of sterile water was added instead of solution C6 to avoid interference in downstream steps, with an incubation period of 5 min at room temperature and centrifuging at 10,000 g for 30 s. This was repeated for a final volume of 100 µL. A procedural blank was regularly included in order to detect possible contamination during sample processing.

2.2.3 Sodium Acetate Precipitation and Cleanup of Nucleic Acids

A sodium acetate precipitation cleanup was used to further purify the DNA for PCR. Twenty µL of 3 M sodium acetate solution (pH 5.2) and 400 µL of cold isopropanol were added to the 100 µL of DNA. The tube was mixed well by inverting several times and placed in the freezer at 20°C for at least 1 h to allow the DNA to precipitate. Samples were centrifuged at 12,000 rcf for 10 min and the liquid was pipetted away from the pellet and discarded. The pellet was washed with 100 µL of 70% ethanol and centrifuged at 12,000 rcf for 5 min. The liquid was discarded, and the pellet washed with 70% ethanol a second time with the same centrifugation step. The pellets were dried on a heat block at 45 °C for 5 min. The DNA was resuspended in 20 µL sterile water and rehydrated at 65°C for 1 h. Samples were then pipetted to mix and centrifuged at 8,000 rcf for 2 min. The pellet was discarded, and the supernatant kept at -20°C.

2.2.4 Test PCR Amplification

A NanoDrop spectrophotometer (NanoDrop products) was used to measure DNA concentrations but due to inconsistent results, PCR was used to identify samples that required dilution before being sent for sequencing, as indicated by a failed PCR test. Samples were sent for NGS after the sodium acetate precipitation cleanup; PCR was simply used to ensure samples could be sequenced. The ITS region was amplified by using the forward primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and the reverse primer ITS4 (5'TCCTCCGCTTATTGATATGC-3') (White et al. 1990). The master mix used for PCR contained 4 µL of 5X Phusion HF Buffer, 0.4 µL of 200 µM dNTPs, 0.8 µL of each primer (0.5 µM) and 0.2 µL of Phusion High-Fidelity DNA Polymerase (2 U/µL) (Thermo Fisher Scientific). One µL of template DNA was added for a 20 µL

reaction volume in 200 μL individual PCR tubes (Fisher Scientific). The amplification conditions consisted of 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s, followed by a final extension step at 72°C for 10 min. Electrophoresis was used to qualitatively evaluate the DNA before sending it for sequencing by using a 1% agarose gel prepared with GelRed (VWR International), and photographed under using a transilluminator.

2.2.5 Next-Generation Sequencing

NGS sequencing of the extracted DNA was performed by Julie Chapados at the Molecular Technologies Laboratory at the AAFC Ottawa Research and Development Centre using the Illumina MiSeq platform. The ITS1 region was amplified from the DNA extracted from the silage samples (2.2.3) using fusion primers (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG(0-3N)(GGAAGTAAAAGTCGTAACAAGG)-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG(0-3N)(GCTGCGTTCTTCATCGATGC)-3') and purified using AMPure beads. The amplicons were normalized using SequalPrep plates (Life Technologies) before a second round of amplification was performed to attach Nextera XT indexes. The purification and normalization were repeated, and library sizes were determined using the Agilent TapeStation D1000. The molar concentration of DNA from each sample was determined by performing both Qubit High Sensitivity DNA assay and KAPA Library Quantification qPCR. Equal quantities of DNA from each sample were loaded on the Illumina MiSeq instrument, and sequencing was performed using 2x300 cycles (600v3) with 25% PhiX

Control Library. Finally, the resulting fastq files were demultiplexed (Chapados, personal communication). All 93 silage samples collected were sequenced.

2.2.5 Bioinformatic Analysis

Analysis of the raw Illumina fastq files was performed using the sequencing pipeline QIIME 2 (version 2019.10) (Bolyen et al. 2019) and following the "Moving Pictures" tutorial (Qiime 2 docs. 2020). A summary of the demultiplexed results produced information on the number of sequences per sample and quality distribution across the reads. The sample reads were trimmed to 209 base pairs after considering this summary in order to remove sequences that would affect the quality of the results obtained if left in the analysis. Quality control of the raw fastq files through the removal of chimeric sequences and correction of errors in the sequencing data were performed using a clustering-free Divisive Amplicon Denoising Algorithm (DADA2) (Callahan et al. 2016). This generated a feature table with Amplicon Sequence Variants (ASVs), which contains the frequencies of each unique sequence in each sample in the dataset, which were used instead of operational taxonomic units after considering Callahan et al. (2017). Next, a multiple sequence alignment was made using the Mafft program (Katoh et al. 2002) and a phylogenetic tree with FastTree (Price et al. 2010) to relate the features to one another. An alpha rarefaction analysis using Faith's phylogenetic diversity confirmed that adequate richness of the samples had been fully observed and adequate sequencing performed as indicated by the rarefaction plot where the samples approached a slope of zero on the x axis. Taxonomic analysis of the sequences was generated by using a Naïve-Bayes classifier trained to the fungal ITS reference database UNITE (User-friendly Nordic ITS Ectomycorrhiza Database) (version 8.0, 02.02.2019 at the 99% threshold) (Kõljalg et al. 2013; UNITE Community 2019). The 20 most abundant ASV

features of all silage samples combined were individually taxonomically classified using the National Centre for Biotechnology Information's (NCBI) basic local alignment search tool (BLAST) using the BLASTN algorithm in GenBank. This number 20 was chosen as a manageable number to examine the taxonomical classification in greater detail. The top 100 sequences on GenBank were reviewed with a specific focus placed on the e-value (the number of expected hits that would be found by chance, with a smaller number indicating a more significant match). No specific e-value range was assigned as the e-values obtained were all of high specificity, however a conservative approach was taken when taxonomically classifying certain features due to ambiguities in the BLAST results.

Differential abundances between the samples was achieved using the analysis of composition of microbiomes (ANCOM) (Mandal et al. 2015). This approach was used to understand whether ASVs or taxa are more or less abundant in different sample groups using the Feature Table which contains the frequencies of features in each sample (QIIME 2019). ANCOM is a compositional approach that does not allow zeros since it is a method that uses a log-transform or a ratio (QIIME 2019), so a pseudocount of one was added to every value to remove the zeros from the Feature Table. The null hypothesis when conducting ANCOM is that the average abundance of the feature in all groups are the same (Akorli et al. 2019). ANCOM does not provide a *p*-value because it is based on the ratio between tests; instead, ANCOM produces a volcano plot which relates the ANCOM W statistic to the CLR (center log transform) for the groups (QIIME 2019). The CLR (also known as the f-score) measures the strength of the difference of a feature between groups with a higher score reflecting a higher likelihood that the null hypothesis can be rejected. The W statistics denote the number of times a feature is significantly

different across groups (Akorli et al. 2019). ANCOM provides the percentile abundances of features by groups (storage type or region). Only 100 percentile abundances were included in the tables with ANCOM data. ANCOM was used to reveal significant features among storage types within regions and between regions.

2.2.6 Mycotoxin Analysis

All 2018 and 2019 silage samples were sent for mycotoxin analysis at Actlabs in Ancaster, Ontario. This lab used LC-MS/MS to detect 16 different mycotoxins: deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, fumonisin B1, fumonisin B2, HT-2, T-2, ochratoxin A, zearalenone, diacetoxyscirpenol, sterigmatocystin and mycophenolic acid. The results for 2019 silage were not yet available at the time of submitting this thesis, so only information from 2018 is presented.

2.3 Results and Discussion

2.3.1 Next-Generation Sequencing Results of DNA Extracted From Silage

The 2018 silage samples contained a total of 6,866 features (ASVs) with a total frequency of 10,090,258. Samples ranged from having 495 to 400,436 features with a mean of 108,497 features. The minimum length of the sequences was 188 base pairs with a maximum of 364 and a mean of 277. Supplemental information on the total number of reads and ASVs can be found in Table A1 of the appendix.

Of the 6,866 features identified, 79.5% belonged to the Kingdom Fungi, 11.4% plant were assigned to Kingdom Plantae, and 9.1% belonged to other groups.
In Kingdom Fungi, 52.4% features were ascomycetes, 26.9% were basidiomycetes, 1.6% were Mucormycota, 5.4% belonged to other phyla, and 13.7% could not be classified

further. Among ascomycetes, 83.% were filamentous fungi, 11.5% were ascomycetous yeasts (Saccharomycetes), and 4.7% could not be classified further. Among basidiomycetes, 51.3% were basidiomycetous yeasts (Tremellomycetes 77% and Microbotryomycetes 23%), 39.3% were filamentous fungi and 9.4% could not be classified further. The 20 most abundant ASV features of all silage samples combined were individually taxonomically classified with information presented in Tables 2.1 and 2.2.

Table 2.1. The 20 most abundant ASVs in 2018 silage produced with DADA2 using QIIME2 and taxonomy produced using BLAST.

#	Kingdom	Phylum	Class	Order	Family	Genus	Species
1	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Monascus*	*Monascus* sp.
2	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Pichia*	*Pichia anomala*
3	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Pichia*	*Pichia kudriavzevii*
4	Plantae	Spermatophyta	Dicotyledonae	Fabales	Fabaceae	*Medicago*	*Medicago sativa*
5	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium* sp.
6	Fungi	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Mrakiaceae	*Leucosporidium*	*Leucosporidium frigidum*
7	Fungi	Ascomycota	Eurotiomycetes	Onygenales	Ascosphaeraceae	*Ascosphaera*	*Ascosphaera atra*
8	Fungi	Basidiomycota	Microbotryomycetes	Leucosporidiales	Leucosporidiaceae	*Leucosporidium*	*Leucosporidium* sp.
9	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Debaryomyces*	*Debaryomyces hansenii*
10	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium* sp.
11	Plantae	Spermatophyta	Monocotyledonae	Poales	Poaceae	*Phleum*	*Phleum pratense*
12	Fungi	Basidiomycota	Tremellomycetes	-	-	-	Tremellomycetes sp.
13	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium delicatulum*
14	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Pichia*	*Pichia kudriavzevii*
15	Fungi	Basidiomycota	Tremellomycetes	-	-	-	Tremellomycetes sp.
16	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Ascochyta*	*Ascochyta* sp.
17	Fungi	Basidiomycota	Tremellomycetes	-	-	-	Tremellomycetes sp.
18	Fungi	Basidiomycota	Tremellomycetes	-	-	-	Tremellomycetes sp.
19	Fungi	Basidiomycota	Wallemiomycetes	Wallemiales	Wallemiaceae	*Wallemia*	*Wallemia canadensis*
20	Plantae	Spermatophyta	Dicotyledonae	Fabales	Fabaceae	*Trifolium*	*Trifolium pratense*

(-) indicates that the feature could not be confidently classified further

Table 2.2. Growth form, total frequency, relative abundance and BLAST results of 20 most abundant ASVs in 2018 silage.

#	Organism	Growth Form	TF	RA	BF	E	SS
1	*Monascus* sp.	Mould	1 479 989	14.7	60	9e-132	100
2	Pichia anomala	Yeast	1 034 099	10.2	68	4e-145	99.65
3	*Pichia kudriavzevii*	Yeast	403 363	4.00	57	4e-98	99.52
4	*Medicago sativa*	Plant	344 018	3.41	63	7e-174	99.13
5	*Penicillium* sp.	Mould	285 316	2.82	60	3e-141	100
6	*Leucosporidium frigidum*	Yeast	273 569	2.71	63	5e-134	100
7	*Ascosphaera atra*	Mould	226 447	2.24	46	1e-151	99.67
8	*Leucosporidium* sp.	Yeast	212 921	2.11	47	3e-113	97.63
9	*Debaryomyces hansenii*	Yeast	195 068	1.93	48	5e-155	100
10	*Cladosporium* sp.	Mould	165 165	1.64	74	1e-130	100
11	*Phleum pratense*	Plant	133 741	1.33	58	3e-162	99.07
12	Tremellomycetes sp.	Yeast	120 198	1.19	58	5e-119	100
13	*Cladosporium delicatulum*	Mould	112 923	1.12	65	3e-131	100
14	*Pichia kudriavzevii*	Yeast	111 261	1.10	19	1e-97	99.51
15	Tremellomycetes sp.	Yeast	110 404	1.09	64	2e-118	99.58
16	*Ascochyta* sp.	Mould	108 919	1.08	51	1e-120	99.59
17	Tremellomycetes sp.	Yeast	108 038	1.07	57	2e-117	99.58
18	Tremellomycetes sp.	Yeast	106 680	1.06	59	9e-137	100
19	*Wallemia canadensis*	Mould	105 177	1.04	28	3e-106	100
20	*Trifolium pratense*	Plant	102 280	1.01	38	3e-172	99.12

TF = total frequency of features, RA= relative abundance (%), BF= by-sample frequency, E= expect value (e-value) SS= similarity score

While

Pichia anomala (synonyms: *Wickerhamomyces anomalus* and *Hansenula anomala*) was the second most common feature (Tables 2.1 and 2.2). This ascomycetous yeast has the ability to grow across a range of pH and temperature conditions, as well as in an anaerobic environment (Fredlund et al. 2002). *Pichia anomala* was found to be a prevalent yeast in corn silage and high moisture corn (Santos et al. 2017) and was also present in grass silage (O'Brien et al. 2007a; O'Brien et al. 2007b; O'Brien et al. 2008; Li and Nishino 2011a).

Pichia anomala has been used as a biocontrol agent of *Penicillium roqueforti*, another common fungal contaminant, in high-moisture cereals (Petersoon and Schnürer 1998; Druvefors et al. 2002; Fredlund et al. 2002). The use of *Pichia anomala* as a biocontrol agent in silage may warrant further investigation as the control of *Pencillium roqueforti* is favourable due to its mycotoxin production capabilities, but it may not be worthwhile due to the role the yeast plays in aerobic deterioration. Perhaps there are better alternatives available such as the use of an inoculant to reduce aerobic deterioration which is described in Chapter 3.

Pichia kudriavzevii was the 3rd and 14th most common feature in the silage (Tables 2.1 and 2.2), appearing twice in the 20 most abundant ASVs. BLAST results indicated high quality e-values and similarity scores from both features. It is unknown if these two features are the same species; they could be the same species but have different environmental or regional preferences, or they could be two closely related species and an appropriate match in GenBank is not yet available.

Pichia kudriavzevii has been isolated from deteriorating silage (Li and Nishino 2011b; Wang et al. 2018). Li and Nishino (2011b) suggested that *Pichia kudriavzevii* does not play a role in aerobic deterioration since it was detectable in corn silage inoculated with *Lactobacillus buchneri* that did not exhibit signs of aerobic deterioration. However, Wang et al. (2018) reported this yeast to be capable of assimilating lactic acid and its inhibition in silage has been associated with enhanced aerobic stability.

Members of the genus *Leucosporidium* were the sixth and eighth most abundant features respectively (Tables 2.1 and 2.2). *Leucosporidium frigidum* (also known as *Mrakia frigida*) was observed in 70% of samples, but little information is available on

this yeast. It has been found in frozen beef (Kabisch et al. 2016), but there are no reports of it in silage. It is possible that the fungi in the silage came from the field and are dead, but the DNA has not degraded yet and pose no risk. *Leucosporidium* sp. was observed in 52% of samples but the species could not confidently be identified beyond the genus level. It is possible that it is *Leucosporidium scottii*, an aerobic basidiomycetous yeast that has been isolated from soil (Middelhoven and Doesburg 2007), but there are no reports of it in silage. *Leucosporidium scottii* had a higher e-value and similarity score than other possible matches in GenBank, but due to ambiguities provided by the GenBank database in terms of number of possible matches to the query sequence, classification of this feature was concluded at the genus level.

The ninth most abundant feature was *Debaryomyces hansenii* (teleomorph of *Candida famata*) (Tables 2.1 and 2.2), a non-fermentative ascomycetous yeast that has been isolated from corn silage (Middelhoven and Baalen 1988; Mansfield and Kuldau 2007), ensiled vegetable crops (Middelhoven et al. 1990), marine environments (Angulo et al. 2019) as well as dairy products where it caused the pH of cheese to rise by consuming lactate and amino acids (Monnet et al. 2015).

Yeasts belonging to the class Tremellomycetes that could not be confidently classified further were the 12th, 15th, 17th, and 18th most abundant features (Tables 2.1 and 2.2). Tremellomycetes spp. have been observed in silage (Duniere et al. 2017; Xu et al. 2019). The 12th most common feature could be the basidiomycetous yeast, *Vishniacozyma victoriae* (also known as *Cryptococcus victoriae*) which has been found in wheat (Jiang et al. 2020; Schiro et al. 2019; Rojas et al. 2020). The 17th most common feature also appeared to be *Vishniacozyma victoriae*. The BLAST searches were further

reviewed to better understand why these two features could potentially be the same species. These two features both have small e-values indicating high quality matches in the GenBank database. Contrary to the BLAST searches performed on *Pichia kudriavzevii*, BLAST was not as confident when identifying *Vishniacozyma victoriae* (Tables 2.1 and 2.2) as indicated by a somewhat weaker e-value, but this could be a similar situation; these two features could be the same species but have different environmental or regional preferences, or they could be closely related but an appropriate match in GenBank is not yet available.

The 18th most common feature in this study could possibly be *Vishniacozyma tephrensis* (also known as *Cryptococcus tephrensis*) which been found in soil (Vishniac 2002) and wheat (Schiro et al. 2019; Rojas et al. 2020), while the 15th feature could not be confidently taxonomically classified further than the class of Tremellomycetes.

While yeasts do not produce mycotoxins, they are the main initiators of aerobic deterioration (Pahlow et al. 2003). Upon oxygen ingress, yeasts and the spores of certain moulds multiply causing major chemical changes by reducing lactic acid content resulting in an elevated pH and a decrease in nutritional value of the silage (Pahlow et al. 2003). The growth of moulds is usually observed after yeasts due to their lower acid-tolerance and greater time needed to multiply (Ávila and Carvalho 2019)

Yeasts responsible for aerobic deterioration have been divided into two physiological groups: the sugar utilizers, and the acid-utilizers which include species of *Canidia* and *Hansenula* (*Pichia*) (Moon and Ely 1979; Woolford 1990; Pahlow et al. 2003). Yeasts belonging to the genera *Candida* and *Pichia* dominate when silage is exposed to oxygen due to their lactate-assimilating ability and strong affinity for glucose

(Pahlow et al. 2003). The population of yeasts able to metabolize lactic acid (lactate-utilizing yeasts) when exposed to oxygen will dictate whether silage will deteriorate (Woolford 1990) as a high population of yeasts does not always mean silage deterioration will occur (Pahlow 1982; Jonsson and Pahlow 1984).

The yeasts present in this study can be categorized by their potential to cause aerobic deterioration in silage. While *Pichia anomala* and *Pichia kudriavzevii* are not perceived as a health risk since yeasts do not produce mycotoxins (Fleet 1992), these yeasts may have played a significant role in aerobic deterioration. Santos et al. (2017) reported *P. anomala* to be physiologically versatile with its ability to grow on sucrose, lactic acid and glucose. *Pichia kudriavzevii* is described as a multi-stress-tolerant yeast able to ferment glucose to ethanol under acid, salt or heat stress **(**Isono et al. 2012). These properties combined with its ability to assimilate lactic acid make it a feature of concern. While the teleomorph of *Debaryomyces hansenii* (*C. famata*) belongs to the genus *Candida*, and Middelhoven and Baalen (1988) observed it to be a less frequently occurring yeast species in corn silage that assimilated lactic acid, Middelhoven et al. (1990) reported *C. famata* to be prevalent in ensiled vegetable crops, but it did not play a role in aerobic spoilage of the silage.

The other yeasts in this study may be less concerning; Xin and Zhou (2007) isolated *Mrakia frigida* from Antarctic soil and determined it capable of fermentation of sucrose but this process was delayed and its assimilation of DL-lactate to be variable. *Leucosporidium scottii* is able to assimilate glucose, but rarely assimilates lactose and does not assimilate DL-lactic acid (Fell et al. 1969). The potential role of Tremellomycetes spp. in aerobic deterioration is unclear; Carrasco et al. (2012) reported

Cryptococcus victoriae to be able to assimilate lactic acid and D-lactose whereas Vishniac (2002) reported the assimilation of lactose by *Cryptococcus tephrenis* to be weak or delayed and that DL-lactate was not significantly assimilated.

In regard to the yeasts present in these silage samples, *Pichia anomala* and *Pichia kudriavzevii* are of particular concern due to their high abundance as well as the role they play in aerobic deterioration of silage with its fermentative and lactate-assimilating abilities. The remaining yeasts may play a smaller role in aerobic deterioration.

The most common feature in this study was an ascomycetous mould belonging to the genus *Monascus* and found in 65% of samples (Tables 2.1 and 2.2). A BLAST search could not confidently taxonomically classify this feature down to the species level, but it is possible that it was *Monascus ruber* or *Monascus purpureus.* Inglis et al. (1999) reported *M*. *ruber* to be a predominant filamentous fungus recovered in barley silage in Western Canada while Schnewuis et al. (2000) and Malekinejad et al. (2015) found *M*. *ruber* to be isolated less frequently than other fungi in grass and corn silage. This potentially toxigenic fungus was also isolated from corn silage by Schneweis et al. (2001) and Garon et al. (2006) where it was producing the secondary metabolite citrinin. *M. purpureus* has also been isolated from silage (Forwood et al. 2019) and is capable of producing citrinin (Blanc et al. 1995), a nephrotoxic mycotoxin produced by certain species of the genera *Aspergillus*, *Penicillium* and *Monascus* (EFSA 2012). Its mycotoxin development usually occurs in storage and is prevalent in stored grains but also in other food products (EFSA 2012). While there are no experimental data available on the systemic toxic effects of this mycotoxin in ruminants, it is predicted that the forestomachs of ruminants are able to degrade and metabolize citrinin, but this function may impair

rumen flora (EFSA 2012). The gastrointestinal absorption of citrinin may occur due to the phenolic and carboxylic groups on molecules of citrinin which give them acidic and weakly hydrophilic properties (Yiannikouris and Jouany 2002).

The fifth most common feature was an ascomycetous species belonging to the genus *Penicillium* (Tables 2.1 and 2.2). This feature could not be confidently classified further based on the BLAST search performed. It is possible that it is part of the *Penicillium roqueforti* group that consists of three species: *P. roqueforti* Thom, *P. carneum* Frisvad, and *P. paneum* Frisvad. (Boysen et al. 1996). *P. roqueforti* was the predominant fungus isolated from corn and grass silage in Germany (Auerbach et al. 1998) and The Netherlands (Malekinejad et al. 2015), and grass silage in Ireland (O'Brien et al. 2005, 2006, 2007a, 2007b). Boysen et al. (2000) suggested that *P. roqueforti* thrives on silage due to the low oxygen level and its ability to grow on organic acids. This utilization of silage acids results in a rise in pH which encourages the growth of other undesirable microorganisms (O'Brien et al. 2008). *P. paneum* has been isolated less frequently from silage (Boysen et al. 2000; Sumarah et al. 2006; O'Brien et al. 2006) and *P. carneum* even less frequently (Nielsen et al. 2006).

The species that make up the *P. roqueforti* group produce different secondary metabolites. Roquefortine C, patulin and andrastin A are produced by all three species, while mycophenolic acid is produced by *P. roqueforti* and *P. carneum*, and Penicillin Roquefort Toxin (PR toxin) and penicillic acid by *P. roqueforti* (Frisvad and Filtenborg 1989; Frisvad et al. 2004). *P. roqueforti*-contaminated feed has been linked to neurologic disorders in farm animals (Malekinejad et al. 2015) while mycophenolic acid has been

found to have immunosuppressive effects (Bentley 2000) and the toxicological properties of andrastin A are unknown (O'Brien et al. 2006).

The seventh most common feature was *Ascosphaera atra* (Tables 2.1 and 2.2**)**, a saprotrophic fungus normally associated with solitary bees and found growing on pollen (Wynns et al. 2013) but it was found growing in grass silage by Skou (1986). It is difficult to understand what ecological role, if any, this fungus may have in silage as there has been little research done on it.

A *Cladosporium* sp. was the 10th most common feature (Tables 2.1 and 2.2) and could be *Cladosporium herbarum*, a mould belonging to the phylum Ascomycota that is ubiquitous in the environment. It is a major cause of fungal allergies and has been linked to the development, persistence, and severity of asthma (Knutsen et al. 2012). It has been found in hay (Kotimaa 1990), barley silage (Inglis et al. 1999) and corn silage (Storm et al. 2010). There are no reports of the genus *Cladosporium* producing mycotoxins. *Cladosporium delicatulum* was the 13th most common feature and has been observed in cereals (Boutigny et al. 2019; Schiro et al. 2019), but has not been detected in silage.

The 16th most common feature in this study belonged to the genus *Ascochyta* (Tables 2.1 and 2.2) and could be *Phoma medicaginis* (synonym *Ascochyta medicaginicol*), an ascomycetous mould that is prevalent in temperate regions of North America and Europe. *Phoma medicagini*, Malbr. & Roum. is responsible for causing spring black stem and leaf spot the most significant disease of alfalfa (Castell-Miller et al. 2007). Research on forage diseases present in Newfoundland revealed *Phoma medicaginis* Malbr. & Roum. to be a frequent pathogen (Compton et al. 2019).

The 19th most common feature was *Wallemia canadensis* (Tables 2.1 and 2.2), a basidiomycetous mould, which has been isolated from house dust, soil and cat walks in silos (Jančič et al. 2015). Members of the genus *Wallemia* are adapted to low water activity and can thrive in high sugar or salt environments (Kunčič et al. 2015). *Wallemia canadensis* is capable of producing the secondary metabolites walleminol, walleminone and wallimidione (Jani et al. 2016). Walleminol has been reported to be of similar toxicity to citrinin and penicillic acid (Wood 1990), but there is very little other information on these secondary metabolites.

The moulds of particular concern isolated in this study are those capable of producing secondary metabolites: *Monascus* sp., *Penicillium* sp. and perhaps *Wallemia canadensis*. There was a high abundance of *Monascus* sp., in particular. The other moulds isolated in this study in the 20 most abundant ASVs are less concerning but likely affect the nutritive value of the silage. Kelman (2016) also reported *Monascus ruber* and members of the *Pencillium* genus to be the most commonly isolated species in a cool-climate silage study in Canada. These cold- and acid-tolerant genera were also the predominant mycotoxin-producing fungi (Kelman 2016).

Silage is heterogenous in nature with differences in chemical and microbiological composition as well as chemical properties within a plot, a plant, a sward and especially within a silo (Pahlow et al. 2003). DNA from the kingdom Plantae was identified (Tables 2.1 and 2.2) because the ITS region is also used to identify eukaryotes, beyond just fungi, and the primers used in this study are general enough that they were able to bind and amplify plant DNA. A considerable quantity of plant DNA was found, but fungal DNA dominated. Plant DNA was not the most abundant type of DNA present even though

silage would contain much more plant material than fungal material on a gram for gram basis due to the degradation of plant material during ensiling. More degradation of plant material within a sample could be due to a more complete fermentation during the ensilage period.

Phleum pratense L. (common name timothy) was identified as the 11th most common feature and is a grass grown across Canada. The legumes *Medicago sativa* (common name alfalfa) and *Trifolium pratense* (common name red clover) were the 4th and 20th most abundant features respectively. These legumes are popular choices on Canadian dairy farms for their high protein content. Silage samples collected were supposed to consist of alfalfa-grass mixtures with little or no red clover. The inclusion of red clover in these samples could be because it is difficult to know what field is being sampled, particularly in tower and bunker silos, in comparison to bales which are commonly labelled with the field identification. Red clover could appear in a field even if alfalfa is the only seeded legume if it is naturally occurring in the seed bank and due to seed carryover from other years. Red clover seeds can also be spread through manure.

2.3.2 Comparison of Features in Storage Types and Regions Using ANCOM

Table 2.3. Significant features in storage types in Atlantic Canada in 2018 using ANCOM and BLAST.

Organism	Growth Form	W	E	SS	Bale n= 20	Bunker n= 19	Tower n= 20
Debaryomyces hansenii	Yeast	4001	5e-155	100	39.0	1,069.0	21,820.0
Leucosporidium sp.	Yeast	3981	3e-113	97.63	1.0	4,166.0	36,976.0
Pichia kudriavzevii	Yeast	3944	4e-98	99.52	20,328.0	75,675.0	1,965.0
Cladosporium sp.	Mould	3938	1e-130	100	1.0	1,682.0	13,721.0
Phleum pratense	Plant	3930	1e-158	99.07	14,967.0	107.0	110.0
Filobasidium uniguttulatum	Yeast	3901	2e-118	100	1.0	66.0	8,390.0
Candida sp.	Yeast	3668	8e-148	100	1.0	242.0	14,469.0

W= W statistic, E= expect value (e-value), SS= similarity score, (-) = no samples were collected from this storage type in this province, 1.0 is a pseudo count meaning this feature was not present in the samples.
Samples were only collected from MB in the Western Canada region in 2018 and therefore a regional ANCOM was not performed. There were no significant features present in the Central region.

Debaryomyces hansenii was higher in tower samples followed by bunker and bales (Table 2.3). This non-fermentative yeast was identified as the ninth most abundant feature in 2018 for all the silage samples (Tables 2.1 and 2.2). *Leucosporidium* sp. was higher in towers followed by bunkers (Table 2.3). This feature was the eighth most abundant feature in all the silage samples (Tables 2.1 and 2.2). The species *Pichia kudriavzevii* was highest in bunkers, followed by bales and towers (Table 2.3). This is the same feature that was the third most abundant ASV in 2018 samples. A species belonging to the genus *Candida* was high in tower with a much lower amount in bunkers (Table 2.3). *Candida* spp. have frequently been isolated from silage (Li and Nishino 2011b; Wang et al. 2018), raw milk (Lavoie et al. 2012) and cheese (Tofalo et al. 2014). The basidiomycetous yeast, *Filobasidium uniguttulatum*, was particularly high in towers, with a lower abundance in bunkers (Table 2.3). *Filobasidium uniguttulatum* has been isolated from the rhizosphere of corn (Gomes et al. 2003) as well as vacuum-packed beef (Kabisch et al. 2016). *Cladosporium* sp. was high in towers, lower in bunkers and not significantly found in bales (Table 2.3). This mould was the 10th most common feature in all of the silage samples (Tables 2.1 and 2.2).

A comparison of the storage types in the Atlantic region for significant features yielded three features identified as *Phleum pratense* using BLAST (Table 2.3). This grass was significant in bales and the only significant plant DNA when comparing storage types. One of the features identified as *Phleum pratense* was the 11th most common feature in 2018 for all the silage samples. The presence of three features all identifying as *Phleum pratense* could be due to small variances in the DNA sequences, perhaps due to differences in cultivars, causing QIIME2 to assign them different feature identifications.

Significant Features Present in Regions by Storage Type

Table 2.4. Significant features present in regions by storage type in 2018 using ANCOM and BLAST.

Organism	Growth Form	W	E	SS	Atlantic	Central	Western
			Bale				
					n= 20	n= 6	n= 4
Helotiales sp.	Mould	2,769	4e-140	100	1.0	239.0	3,333.0
			Bunker				
					n=19	n= 8	n= 3
Vishniacozyma carnescens	Yeast	1,957	2e-115	99.58	1.0	145.0	5,214.0
Cystofilobasidium macerans	Yeast	1,941	2e-122	100	1.0	1.0	3,227.0
Filobasidium sp.	Yeast	1,937	1e-99	99.06	1.0	1.0	1,240.0
Tremellomycetes sp.	Yeast	1,930	1e-84	97.91	1.0	1.0	1,298.0
Dioszegia crocea	Yeast	1,875	2e-102	100	129.0	1.0	1,735.0
Sporobolomyces sp.	Yeast	1,871	3e-126	100	1.0	1.0	750.0
Hannaella sp.	Yeast	1,779	8e-112	100	1.0	20.0	222.0
Tremellomycetes sp.	Yeast	1,773	2e-112	98.31	1.0	65.0	456.0
Mrakia sp.	Yeast	1,759	2e-123	97.04	1.0	96.0	2,405.0
			Tower				
					n= 20	n= 9	
Filobasidium magnum	Yeast	407	3e-121	100	580.0	1,083.0	-
Glycine sp.	Plant	81	7e-164	99.08	196.0	1,352.0	-
Tremellomycetes sp.	Yeast	75	2e-118	99.58	138.0	858.0	-

W= W statistic, E= expect value (e-value), SS= similarity score, (-) = no samples were collected from this storage type in this province, 1.0 is a pseudo count meaning this feature was not present in the samples.

The order Helotiales present in bales was higher in Western Canada (Table 2.4) and has been reported in pasture (Mysterud et al. 2007) and soil (Klaubauf et al. 2010). There is very little information on this ascomycetous mould and any associated mycotoxin production. This feature was most abundant in Western Canada, followed by Central region, but not present in Atlantic Canada.

All of the features significantly higher in bunkers belong to the class Tremellomycetes except *Sporobolomyces* (Table 2.4). *Dioszegia crocea* (also known as *Bullera crocea*) has also been isolated from wheat fields (Blixt et al. 2010) and growing on the roots of various plants (Renker et al. 2004) but there are no reports of it in silage. Drouin et al. (2019) reported *Hannaella* to be a main yeast genus in corn silage. The genus *Mrakia* has been isolated from camel milk (Amrouche et al. 2019) but there are no reports of it in silage.

The genus *Filobasidium* has been reported to be an abundant genus in fresh corn silage and declines with ensiling time (Xu et al. 2019). The Tremellomycetes sp. could not be confidently identified beyond the class level using BLAST. *Cystofilobasidium macerans* has been isolated from soil (Glushakova et al. 2015) and vacuumed-packed beef (Kabisch et al. 2016) but there are no reports of it in silage. *Vishniacozyma carnescens* has been isolated from soil (Abdullabekova et al. 2017). While there are no reports of this species in silage, other species in this genus have been isolated from corn and rice (Yurkov and Kurtzman 2019).

The abundance of Tremellomycetes in silage has been reported to differ with sampling time; Tremellomycetes were abundant fungi in fresh forage but declined with storage time (Xu et al. 2019) and aerobic exposure (Duniere et al. 2017). The genus

Sporobolomyces has been isolated from corn silage (Xu et al. 2019) and has also been reported to decline with ensiling (Xu et al. 2019). The high presence of these features in bunkers from Western Canada bunkers and not in other regions except for low levels with some features likely due to the sampling time in MB where silage was sampled in the summer months shortly after harvest compared to the winter months in the remaining provinces.

The two features in towers belonging to the class Tremellomycetes were more abundant in the Central region than Atlantic Canada (Table 2.4). *Filobasidium magnum* (formerly *Cryptococcus magnus*) has been found in camel milk (Amrouche et al. 2019). Another feature could not be identified further than the class level.

A feature belonging to the genus *Glycine* was more abundant in the central region in tower samples (Table 2.4). This feature is likely *Glycine max* (common name soybean) which is an annual legume grown across Canada. Soybean meal is produced as a by-product when extracting oil from soybeans and is the most commonly used protein supplement in dairy rations (Bernard 2011). The presence of soybean in the silage samples likely occurred due to contamination on the conveyer belt used to unload feed as the same belt is often used for both soymeal and forage (Lafrenière, personal communication). Soybean contamination could have also originated from dust deposited on the belt as most silos were in the surrounding area (Lafrenière, personal communication). Only a tiny amount of soybean would have needed to be present as PCR, a component of DNA sequencing, is very sensitive and is able to detect even small amounts of organism-specific DNA (Vural 2010).

The early sampling time in Manitoba seemed to affect the fungi present in the silage, with many of the features present reported to decline with ensiling time. Undi et al. (1997) observed field fungi including *Phoma*, *Alternaria*, *Cladosporium* spp. and most yeasts to be eliminated within 8 days of storage in alfalfa hay. Research on the seasonal variations in the microbiology of corn silage between three and eleven months after ensiling in Denmark by Storm et al. (2010) revealed that numbers of filamentous fungi were highest five to seven months after ensiling and the lowest after 11 months. The decrease in quantity after seven months in storage could be due to a reduction in the total number of fungal propagules or reduced viability of the propagules (Storm et al. 2010). While many of the features present or in higher abundance in Manitoba were yeasts and not filamentous fungi, the growth of yeasts initiates aerobic deterioration through a rise in pH, which creates a more suitable environment for the growth of filamentous fungi (Pahlow et al. 2003).

The yeasts that are most concerning based on the ANCOM results presented in Tables 2.3 and 2.4 are those that have lactate-assimilating abilities which contribute to aerobic deterioration of silage. *Pichia kudriavzevii* and *Candida* sp. are of particular concern as described previously in this chapter. The species belonging to the genus *Sporobolomyces* could be *Sporobolomyces roseus*. Its assimilation of DL-lactate was active in some strains and latent in others (Yamazaki and Komagata 1983). Yeasts that are less concerning include *Debaryomyces hansenii*, *Leucosporidium* sp. and Tremellomycetes spp. based on the information provided previously in this chapter.

The plastic covering bunkers and bales is susceptible to holes for oxygen to gain entry and initiate the growth of *Pichia kudriavzevii*. The high presence of *Candida* sp. in

towers (Table 2.3) suggests that oxygen is gaining entry to the silo or that oxygen was not used up in the respiration phase. In top unloading tower silos, forage is removed from the top layer. This unloading face has continual exposure to oxygen which encourages the growth of aerobic organisms which consume dry matter and leads to the release of heat and moisture (Holmes 2000). To reduce the risk of a spoilage layer, the face of the tower silo must have an adequate removal rate (Holmes 2000).

All of the towers sampled in this study were top unloading conventional concrete silos which may not be very airtight. Oxygen could also enter towers between harvests when additional forage is added to the silo, and especially if the silo is not closed between harvests. According to Moore et al. (2020), the upper surface of concrete tower silos is usually left open to the air and the top 1 m of loose silage may be affected by spoilage. Another large factor to consider is the moisture of the forage being ensiled; dry forage does not pack as well, and air pockets could exist within the silo. The high prevalence of yeast such as *Candida* sp. could also be attributed to slow filling of the tower as well as a slow feed out rate.

Other less concerning yeasts including *Debaryomyces hansenii, Leucosporidium* sp. and *Filobasidium uniguttulatum* were most abundant in towers. *Debaryomyces hansenii* cannot grow in the absence of oxygen (Rozpędowska et al. 2011), further suggesting that the high presence of this yeast indicates that oxygen is in contact with the silage in some way, whether this be through oxygen pockets within the silage, from slow consolidating (packing) or a slow feed out rate.

There are many benefits associated with feeding out of tower silos; most tower silos are attached to a barn and can be easily fed out of without burning diesel fuel and do

not require plastic like bunker silos. Conventional tower silos are an aging storage system in Canada with many of them no longer airtight as indicated by the high yeast numbers. Producers should consider applying shock crete to their silos to make them more airtight or consider alternate storage types to better preserve forage quality.

High fungal abundance in bunkers could be due to the equipment used during packing of the silage. The use of self-propelled harvesters or larger equipment often results in silage being transported to the bunker faster, but the packing equipment may not be able to keep up with the amount of silage coming in, making it difficult to remove the oxygen from the bunk and encourage an ideal fermentation. A tower silo does not face this challenge as the forage packs itself, but towers are susceptible to oxygen ingress as top-unloading silos are not covered when they are being fed out of. Bunker silos may need additional equipment and weight when a large volume of forage is being brought to the bunker quickly, in order to pack the silage adequately and remove the oxygen as rapidly as possible. Sufficient removal rates from the face of the bunker silo are also necessary to reduce fungal contaminants.

Cladosporium sp. was most abundant in towers (Table 2.4). Although there is no known mycotoxin production associated with this genus, its high abundance suggests aerobic deterioration could be occurring. Oxygen entry into this storage type could have instigated yeast growth and in turn provided a more favourable environment for mould growth such as *Cladosporium* sp, however, the contamination of forage with these genera likely occurred in the field and its abundance may have been high even if an ideal fermentation had occurred and restricted any further multiplication of the fungi.

There were few significant features when comparing samples between regions and storage types. The lack of differences found are likely attributed to too few samples for each specific comparison and high sample-to-sample variability as a sample could not be categorized to a storage type or region simply by inspecting the fungi in it. The fungal presence and abundance did differ across Canada and is likely attributed to sampling times and regional factors such as management practices. While climatic factors such as temperature, humidity and precipitation were not measured, they are known to differ across the country and likely affected the results obtained.

2.3.3 Comparison of Fungi in Present Study to Other Studies

The second hypothesis established was that similar fungi would be detected in silage as those found in a similar temperate climate, and may include *P. roqueforti, P. paneum*, *Schizophyllum commune* (O'Brien et al. 2008), *Aspergillus fumigatus* (Richard et al. 2009), and yeasts including *Pichia fermentans*, *Pichia anomala*, and *Geotrichum* spp. (O'Brien et al. 2008). *Penicillium* species was the fourth most abundant feature in this study. Although it could not be confidently identified down to the species level, *P. roqueforti* and *P. paneum* are probable. *Pichia anomala* was the most abundant yeast in this study and *Pichia fermentans* was also identified.

Aspergillus fumigatus was only identified in one sample with a relative abundance of 1.814%. Boudra and Morgavi (2005) reported *A. fumigatus* to grow poorly in forage substrates, in particular legumes (red clover and alfalfa), but extensively in cereals including corn. Amigot et al. (2006) had similar findings in their research of mycotoxins on alfalfa and corn silage; *A. fumigatus* was not isolated from alfalfa silage, but was from corn silage.

O'Brien et al. (2005) also compared the fungi found in grass bale silage in Ireland with other countries. They noted that *Schizophyllum commune* was absent from bale silage in Norway (Skaar 1996) whereas *Aspergillus* spp. was not isolated from Irish bales, but were frequently isolated in Norway. Possible explanations for differences in bale mycobiota of grass silage between these two countries included climatic factors and differences in bale management practices. These suggestions could explain why *S. commune* was not identified in this study.

Research on corn silage in Alberta, Canada showed *Geotrichum* spp. to remain undetectable after ensiling, only to increase after 118 days of ensiling (Xu et al. 2019). After aerobic exposure, this genus increased in abundance after 3 days, but declined after 7 days (Xu et al. 2019). Silage samples collected in Western Canada took place before 118 days of ensiling which could explain the lack of *Geotrichum* spp. found. *Geotrichum* may not have been detected in samples east of Western Canada due to climatic factors and differences in silage management as suggested above.

2.3.3. Mycotoxin Incidence and Concentration

Table 2.5. Incidence and concentration of zearalenone and mycophenolic acid in silage.

		Zearalenone Concentration (μg kg^{-1})			Mycophenolic acid Concentration (μg kg^{-1})		
Year	Sample, n	Positive samples	Mean	Maximum	Positive samples	Mean	Maximum
2018	93	1 (1%)	350	350	10 (11%)	1,346	3,480

In 2018, of the 16 mycotoxins that were tested for (deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, fumonisin B1, fumonisin B2, HT-2, T-2, ochratoxin A, diacetoxyscirpenol and sterigmatocystin), only zearalenone and mycophenolic acid were detected (Table

2.5). Similar findings were found in a 2-year Irish national survey on mycotoxins in farm silages (mostly grass) conducted by McElhinney et al. (2015) that tested 300 silage samples for the same mycotoxins in this study, with the exception of 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, diacetoxyscirpenol and sterigmatocystin, and found the mycotoxins were below detectable limits in all samples except zearalenone and mycophenolic acid.

While fumonisin B1 and B2 were not detected, *F. proliferatum* which produces these mycotoxins was detected in 11 samples ranging from 0.040 – 1.650% relative frequency. *Fusarium sporotrichioides,* responsible for the production of T-2 toxin and diacetoxyscirpenol, was present in 15 samples with relative frequencies of 0.082 - 1.399%. *Aspergillus versicolor,* which produces sterigmatocystin, was identified in 17 samples with relative frequencies of 0.046 - 0.401%. These mycotoxin-associated species were identified but toxin production was not above detectable limits.

Forage crops can be infected with pre-harvest fungi belonging to the genera *Fusarium*, *Alternaria*, and *Cladosporium* (Scudamore and Livesey 1998). Once the crop is harvested and ensiled, further mould growth and mycotoxin development is low if oxygen is removed. However, should oxygen gain access to the silage, post-harvest storage moulds from the genera *Penicillium* and *Aspergillus* may proliferate (Scudamore and Livesey 1998). Both pre-harvest (*Fusarium*) and post-harvest (*Penicillium*) moulds were found to be producing mycotoxins in 2018 which was also observed by McElhinney et al. (2015).

In 2018, species of the genus *Fusarium* were present in 50 of the 93 samples (53%) but ZEA was detected in only one sample at a detection limit of 0.003 $\mu g\ kg^{-1}$

(Table 2.5). The sample was taxonomically identified as containing 0.637% *Fusarium*. *Fusarium graminearum*, which is responsible for the production of ZEA, was not identified in the fungal DNA of the silage samples, even though one sample contained ZEA. It is possible that the DNA of *F. graminearum* degraded but the mycotoxin remained. In this study, samples were collected from Manitoba in 2018 at harvest or very soon after (0-65 days). Sample collection dates are provided in the appendix (Table A3). The sample that ZEA was detected in was collected on the same day it was baled and therefore not a fermented sample. There was only one other sample collected on the same day it was harvested, but it did not contain ZEA. The contamination of feed with ZEA has been found to have negative effects on the health of dairy cattle; early abortions in cows have been associated with feed contaminated with infected with 10,000 μg kg^{-1} of ZEA (Kallela and Ettala 1984) and reduced conception rates were observed in heifers that received 12 500 μg kg^{-1} (Weaver et al. 1986). Jones et al. (1994) suggest that ZEA not exceed 250 μg kg^{-1} in the dairy cow diet.

Research in the Czech Republic revealed alfalfa and red clover silage to have ZEA values ranging from 41.18 to 107.23 μg kg^{-1} (Skladanka et al. 2017). Reed and Moore (2009) reported ZEA concentration to be correlated with crude protein and ZEA levels to be higher in legume-dominant stands than mixed grass + legume and grass-dominant stands (22,100, 3,850 and 650 μg kg^{-1} respectively) with a significant difference found between the grass- and legume-dominant silages. Driehuis et al. (2008b) reported grass and wheat silage collected in The Netherlands to contain less mycotoxins than corn silage. Out of 120 grass silage samples, only seven (6%) tested positive for ZEA (detectable limit 25 μg kg^{-1}) with a mean of 93 μg kg^{-1} and maximum

concentrations of 308 μg kg^{-1}. Similarly, Driehuis et al. (2008a) found one sample out of 16 grass samples (6%) to contain ZEA with a concentration of 180 μg kg^{-1}. Low concentrations of ZEA were also detected in three out of 150 grass samples (2%) in Ireland with a mean of 53 μg kg^{-1} (McElhinney et al. 2015).

A Polish study detected ZEA and DON at a frequency of 3% and 37% respectively in grass silage (Panasiuk et al. 2019), while a German study conducted by Korn et al. (2005) detected ZEA in 22% (6-66 μg kg^{-1}) and DON (63-1,290 μg kg^{-1}) in 39% of grass silage samples. Korn et al. (2005) concluded mycotoxin contamination to be low with no increased risk for animal populations and consumers. Driehuis et al. (2008a) frequently observed co-occurrence of DON and ZEA but did not report what harvest the silage was collected from. Weather conditions that encourage *Fusarium* spp. development include moderate temperatures (20-30°C), along with high relative humidity (90%), recurrent precipitation, and air currents (Stanciu et al. 2019). Perhaps these weather conditions were not present for the co-occurrence of DON and ZEA or the development of other *Fusarium*-associated mycotoxins.

While there was only one sample that tested positive for ZEA, it was above the recommended threshold for dairy cattle suggested by Jones et al. (1994) but below the concentrations that have been found to cause early abortions (Kallela and Ettala 1984) and reduced conception rates (Weaver et al. 1986). In Canada, the recommended tolerance level of ZEA in the diets of cattle is 10,000 μg kg^{1} and 1,500 μg kg^{1} if other toxins are present (Government of Canada 2017).

Mycophenolic acid is produced by *Penicillium* species, especially *P. roqueforti.* *Penicillium* sp. was the fifth most abundant feature in this silage study and observed in 60

out of 93 silage samples with ten samples found to be producing MPA (11%) at a detectable limit of 0.003 μg kg^{-1} (Tables 2.1, 2.2, 2.5). Schneweis et al. (2000) reported 36 out of 98 (37%) grass silage samples in Germany to contain MPA with a mean of 2,200 μg kg^{-1} and a range of 21 – 35,000 μg kg^{-1}. Driehuis et al. (2008a) detected MPA in the surface layer, but not from core samples, while Driehuis et al. (2008b) did not find MPA in 120 grass silage samples collected in The Netherlands likely due to a difference in silage use at time of sampling; Driehuis et al. (2008b) collected samples from sealed silages with no air exposure. McElhinney et al. (2015) also reported low levels of MPA. Grass silage samples collected from bales and horizontal bunker silos from Irish farms had low incidence of mycotoxins; seven samples tested positive for MPA in year one (5%) and one in year two (1%) with the highest concentration of MPA being 4,386 μg kg^{-1}. McElhinney et al. (2015) concluded that the risk for animals consuming this silage was generally low. Climatic factors and differences in management practices could be the cause of slight discrepancies in these European studies as suggested by O'Brien et al. (2005)

Information on the effects of MPA on ruminant health are limited. Mohr et al. (2007) investigated the effect of feeding MPA to sheep at varying concentrations up to 300,000 μg daily which is equivalent to 5,400 μg kg^{-1} body weight for 44 days. There were no differences in the general health of the animals, and it was concluded that MPA concentrations occurring naturally in silage have no obvious impact on sheep health. Driehuis et al. (2008a) suggest future research focus on the effect of long-term exposure of animals to mycotoxins with immunosuppressive effects, such as DON and MPA, which may result in an increased susceptibility to infectious diseases. Data on the

carryover of MPA into milk and other dairy products are limited. MPA was not detected in raw and unpasteurized milk and levels in cheese were negligible (Usleber et al. 2008). Driehuis et al. (2008a) concluded that the occurrence of MPA in silage in the concentrations detected in their studies were not a significant concern for the safety of dairy products for consumers.

The data to qualify and quantify the risk of MPA are not available (European Commission 2003). Due to the lack of information on the effect of MPA on immunotoxicity in farm animals, and its occurrence and carry-over into animal products, the significance of this mycotoxin for animal and human health cannot be properly evaluated (European Commission 2003). While the levels of MPA detected in this study are much lower those used in Mohr et al. (2007), the effects of chronic low-level exposure of ruminants to MPA are not known; more research needs to be done before it can be confidently concluded that that the levels of MPA in this study will not result in adverse health issues.

Low levels of mycotoxins detected in this study could be attributed to the type of silage sampled. Higher incidence and greater concentrations of mycotoxins have been detected in other crops in comparison to grass silage, especially corn (Driehuis et al. 2008b; Zachariasova et al. 2014; Panasiuk et al. 2019). Auerbach et al. (1998) observed corn silage to offer more favourable conditions for mycotoxin formation than grass silage. This was also noted by Zachariasova et al. (2014) when comparing corn silage with clover-grass and alfalfa silages; corn silage was contaminated with a wide spectrum of *Fusarium*, *Alternaria*, and *Penicillium* mycotoxins likely due to the leaves and corn cobs being rich in nutrients such as proteins and polysaccharides, providing substrate for

fungi. When considering the effect of mycotoxin exposure on animal health, Panasiuk et al. (2019) suggest that grass silage could be a "safer" option than corn due to its lower toxins.

The low presence of mycotoxins in this study could also be attributed to the cooler weather when the silage samples were harvested. Skladanka et al. (2013) analyzed fresh cut and conserved grass samples for multiple mycotoxins including DON, ZEA, fumonisin, aflatoxin, and T-2 toxin. This research involved cutting grass at the beginning of June, end of July, beginning of October, beginning of November and beginning of December with the first cutting ensiled. DON, ZEA and T-2 were the most prevalent mycotoxins detected. DON content was highest in July, followed by October and November. ZEA content was highest in late July and continued until October. Skladanka et al. (2013) reported low temperatures to reduce the risk of mycotoxins particularly in November and December. In this study, June was found to be a period with low incidence which is the same month that the large majority of the samples collected in this study were harvested.

Greater mycotoxin incidence may not have been observed due to the degradation of mycotoxins during ensiling. Mansfield et al. (2005) observed the degradation of DON during storage perhaps due to microbial agents present in silage that have the ability to degrade or bind this toxin as suggested by He et al. (1992) and El-Nezami et al. (2002). Boudra and Morgavi (2008) reported the concentration of four major *Fusarium* toxins, DON, fumonisin B1 and B2, and ZEA to decline with ensiling, with ZEA disappearing by 50% and DON by up to 100%, whereas Skladanka et al. (2017) reported ensiling to lower the content of DON, but not ZEA. Mansfield and Kuldau (2007) did not detect

viable Fusaria in ensiled corn, implying that *Fusarium* species do not survive ensiling, likely due to low pH combined with low oxygen tension. However, most *Fusarium* mycotoxins in silage are produced in the field (Mansfield and Kuldau 2007) and some toxins such as ZEA are stable in silage (Jensen et al. 2020) suggesting that toxin levels may not decrease much before the silage is fed (Mansfield and Kuldau 2007).

Mycotoxin production in ensiled forage was found to be time dependent. Müller and Amend (1997) inoculated corn silage with *P. roqueforti*, which is able to form MPA, patulin, penicillic acid and PR toxin, for an observation period of 160 days. MPA, patulin, penicillic acid, and PR toxin were detected for the first time at 36, 22-27, 13 and 49 days after ensiling with maximum toxin levels of 3,560, 15,100, 3,060, and 2,170 μg kg^{-1} respectively. The authors observed that toxin levels decreased to a low or non-detectable level with increasing storage time and the probability of detecting these mycotoxins in corn silage under practical conditions of agriculture is low. This observed production pattern of mycotoxins makes successful toxin monitoring in silage difficult and also hinders the evaluation of harmful health effects in animals (Fink-Gremmels 2004). Some toxin levels may still be detectable, depending on the type of *Penicillium* mycotoxin; roquefortine C and mycophenolic acid appear to be stable in silage, while PR-toxin and patulin seem to be unstable (Driehuis 2013).

In regard to this study, changes in mycotoxin profiles are unknown as sampling only occurred once. The timing of sampling could have been before some mycotoxins developed or after some mycotoxins degraded. The sample that contained ZEA was collected on the same day it was ensiled, indicating that mycotoxin formation occurred in the field. The ten samples that contained MPA originated from four samples from

Atlantic Canada and two from ON that were all collected in the winter months, and four samples from MB that were sampled 0, 7, and 29 days after ensiling. It is unknown when the fourth sample was collected. The detection of MPA at an earlier time period than above from Müller and Amend (1997) suggests MPA formation before ensiling, implying it is not only a post-harvest mycotoxin producing mould. Lacey (1989) suggests that the concept between field and storage fungi should be not pressed too far as the boundary between pathogens and saprophytes is blurred and some field fungi are able to grow on dead tissue in storage.

2.4 Conclusion

An in-depth look at the top 20 ASVs revealed the presence of some yeasts associated with aerobic deterioration as well as mycotoxin-associated fungi. The high abundance of *P. anomala* and *P. kudriavzevii* were the most concerning yeasts due to their fermentative and lactate-assimilating abilities. The moulds of most concern included *Monascus* sp. and *Penicillium* sp. due to their possible mycotoxin production.

Tests performed using ANCOM showed some differences in fungi in storage systems in provinces and regions. Many of the features in silage collected from Manitoba were reported to decline with ensiling time and were likely present due to the early sampling time in this province. The comparison of fungi in this study with other studies showed some similarities. Other fungi were not isolated as hypothesized perhaps due to climatic conditions and differences in management practices.

Mycotoxin contamination in the silage samples were low. Mycotoxin analyses determined ZEA to be present in one sample (1%) and MPA in ten samples (11%). While MPA levels in this study were lower than research done in other areas of the world,

additional research is necessary to determine if the levels of MPA in this study are a threat to ruminant health.

Chapter 3: Evaluating the Relationships Between Silage Management and Quality Parameters

3.1 Introduction

Poor management and the contamination with spoilage or pathogenic organisms can reduce the quality of ensiled forages (Adesogan 2018). The ingress of oxygen into silage is the most important factor affecting silage quality as oxygen exposure can encourage yeasts and moulds which affect the nutritive value of the silage and can cause spoilage. According to Pahlow et al. (2003), air will gain access to the silo during the storage period because the silo is never perfectly sealed airtight. Moulds usually occur on the surface layers as a result of poor compaction and/or poor sealing (Woolford 1990). These moulds can produce mycotoxins which have been linked to animal performance and health issues (Adesogan 2018). Good silage management practices, which aid fermentation and inhibit spoilage, can prevent or lessen these negative outcomes (Adesogan 2018).

3.1.1 Storage Type

Numerous management practices are contemplated when making silage. General decisions include the harvest date, harvest DM, and the possible use of an inoculant in the forage harvester or at the storage facility. There are also specific management practices for each storage system; silage quality in bunker silos is influenced by decisions made when filling and feeding out the silo; tower silos also require rapid filling and adequate feed out to reduce spoilage; and different management practices arise when making round bale silage to quickly exclude oxygen and encourage an optimal fermentation.

Baled silage is not chopped to the same extent as forage stored in bunker and tower silos, making it inherently more susceptible to aerobic deterioration (Weinberg and

Ashbell 2003). It also has a higher surface-to-volume ratio with approximately half of the silage within 15 cm of the plastic film where it is more vulnerable to air ingress and mould growth (O'Kiely et al. 2002). While bales have an inherently restricted fermentation, baled silage can be well preserved by quickly applying at least six layers of plastic to exclude air, and continually inspecting the bales until feeding (Coblentz and Atkins 2019). Bales should be wrapped quickly (within 24 h) to exclude oxygen, conserve forage quality and encourage an optimal fermentation (Undersander et al. 1998; Crook et al. 2016).

Harvesting forage at the correct DM is key to producing high quality silage. Forage should be ensiled in the 30-50% DM range with the ideal harvest DM differing with storage type; bunkers should be ensiled at 30-40% DM and towers drier at 40-50% DM (Muck and Shinners 2001). Since bale silage undergoes a reduced fermentation in comparison to chopped silage (Huhnke et al. 1997), it is more at risk for a clostridial fermentation caused by spoilage bacteria and should be ensiled at 5 to 10 percentage points higher in DM (Muck and Shinners 2001) with Clark (2003) suggesting the ideal DM for bales to be 45-60% DM. Conversely, Nicholsen et al. (1991) reported a more desirable fermentation pattern to occur in bales ensiled at 35-41% DM than 46-51% DM as evidenced by a more rapid and lower drop in pH and higher lactic and acetic acids. Coblentz and Atkins (2019) also reported moisture to drive fermentation with wetter bales achieving a more acidic final pH.

Forage ensiled with a low DM will likely lead to a poor fermentation and silage with a high pH value, less lactic acid and acetic acid (Bijelić et al. 2015) and can also cause seepage (effluent) out of the silo (Han et al. 2014). Forage ensiled at a high DM is

difficult to compact creating a longer aerobic process, potentially leading to oxidative losses and the occurrence of mould (Han et al. 2014; Bijelić et al. 2015). O'Brien et al. (2007b) reported low DM to favour the occurrence of *P. fermentans*, *Geotrichum*, *P. roqueforti* and *P. paneum,* and higher DM to encourage the growth of *P. anomala*, *Schizophyllum commune* and mucoraceous moulds.

The production of lactic acid by LAB is most responsible for a decline in pH as it is 10-12 times stronger than the other relevant acids such as acetic acid (Kung et al. 2018). Lactic acid is inversely related to DM with higher lactic acid in lower DM silages. After lactic acid, acetic acid is found in the highest concentration in silage (Kung et al. 2018). As with lactic acid, acetic acid is inversely related to DM content, with acetic acid declining with increasing DM (Kung et al. 2018). Moderate concentrations of acetic acid can improve the aerobic stability of silage by inhibiting spoilage microorganisms through its antifungal properties (Danner et al. 2003; Kung et al. 2018).

Certain *Clostridia* species produce butyric acid with high levels linked to animal health issues such as subclinical ketosis (Vicente et al. 2014). The production of butyric acid caused by a clostridial fermentation usually results in DM losses and depleted nutrients, but it can enhance aerobic stability of silage (Adesogan et al. 2004) due to its strong antifungal characteristics (Kung et al. 2018). Some *Clostridia* spp. are saccharolytic and ferment sugars to butyric acid, while others are proteolytic (Kung et al. 2018). Saccharolytic butyric bacteria rely on sugars and lactic acid for growth to produce n-butyric acid, while proteolytic butyric bacteria use protein as a substrate source to produce i-butyric acid (Lafrenière, personal communication). A slow drop in pH or clostridial activity of silage can lead to excessive protein breakdown and cause high

concentrations of ammonia (Kung and Shaver 2001). Silages that are at risk for higher concentrations of ammonia include those that are not packed tightly or are filled slowly, as well as those ensiled too wet, especially those under 30% DM (Kung and Shaver 2001).

McEniry et al. (2006) and McElhinney et al. (2015) reported bales to have higher pH and lower lactic acid and acetic acid than bunkers and attributed the results to the lower DM of bales. McEniry et al. (2006) reported no differences in butyric acid in bale versus chopped silage when ensiled at 36% and 22% DM respectively. McElhinney et al. (2015) reported bales to have higher butyric acid values in comparison to bunker silos, suggesting excess clostridial activity especially with their elevated pH. In this two-year Irish study, bales were ensiled on average at 29.1% and 32.4% DM, and bunkers at 23.9% and 25.4% DM in years one and two respectively. Forage in this study was ensiled wetter, perhaps due to a more challenging climate to wilt forage. Ammonia levels did not differ between bunkers and bales (McEniry et al. 2006; McElhinney et al. 2015). While lower concentrations of lactic acid and a higher pH can encourage the growth of fungal contaminants, McElhinney et al. (2015) did not observe bales to have higher incidence of MPA than bunkers.

3.1.2 Heating

Heating in silage occurs at ensiling because there is still oxygen trapped in the forage mass. This oxygen is consumed by plant respiration and aerobic bacteria, yeast and mould activity (Gallagher and Stevenson 1976; Coblentz and Hoffman 2008). Aerobic microorganisms consume soluble carbohydrates, resulting in the production of carbon dioxide and heat, causing the temperature of the forage mass to increase. This

process, referred to as the aerobic phase of ensiling, can last for days or even weeks if there is a large amount of oxygen trapped in the forage (Coblentz and Hoffman 2008). Additional heating can occur if oxygen is gaining entry to the storage system, encouraging yeast and mould growth (Gallagher and Stevenson 1976) which reduces lactic acid, causing a rise in pH and decrease in nutritional value (Pahlow et al. 2003).

The occurrence of heat damage in forages is usually the result of changes to forage protein quality due to Maillard reactions (or browning reaction), a heat-induced chemical reaction where certain plant carbohydrates (usually sugars) react with proteins (amino acids) rendering the forage proteins less digestible to dairy animals (Coblentz and Hoffman 2008; Coblentz et al. 2011). While the test to measure heat damaged protein is essentially the same, there are many different terms for this measurement; as the laboratory test measures the amount of CP retained in acid detergent fiber, Coblentz and Hoffman (2008) suggest acid detergent fiber crude protein (ADF-CP) to be the best definition.

Although a certain amount of heat is unavoidable during silage fermentation, management practices can impact the occurrence and extent of heat-damaged protein (Kung et al. 2018). Chopping forage adequately, packing forage well and sealing the silo immediately after filling will ensure the temperature of the forage mass does not increase more than 5 to 8°C above the ambient temperature (Kung et al. 2018). While filling, temperatures in the uppermost layers of the forage mass will be more at risk of reaching higher temperatures (as high as 45 to 60°C) which can lead to heat-damaged protein but should decrease if appropriate packing is performed to remove the air from the mass (Kung et al. 2018).

Silages with higher DM tend to have higher pH values as the silage is drier and ferments less, and typically have more browning, although the rate of browning may be slower (Goering and van Soest 1972; Gallagher and Stevenson 1976). Forage ensiled at higher DM may be more vulnerable to heat damage as drier silages do not pack as well as wetter silages, increasing the susceptibility to oxygen ingress and subsequent heating (Gallagher and Stevenson 1976; Kung et al. 2018). Goering and van Soest (1972) reported silages with limited fermentation as indicated by pH are more prone to heat damage.

3.1.3 Inoculant

LAB can be categorized by their fermentation of glucose into homofermenters and heterofermenters (Muck 2010). Homofermenters are more efficient producers of lactic acid as they produce two moles of lactic acid from one mole of glucose, whereas heterofermenters produce one mole each of lactic acid, carbon dioxide and either ethanol or acetic acid from one mole of glucose (Muck 2010). Although the ensiling process can be successful by relying on epiphytic LAB to begin fermentation and conserve nutrients, inoculants comprising certain strains of LAB have been developed to reduce this reliance (Weinberg et al. 1993) and are the most common additives for making silage (Muck 2010). Homofermentative LAB inoculants quickly and efficiently convert water soluble carbohydrates into large quantities of lactic acid, lowering the pH and allowing the silage to be conserved with lower fermentation losses (Weinberg et al. 1993). *Lactobacillus plantarum* is the most common homofermentative species, but others include *Lactobacillus casei*, numerous *Pediococcus* species and *Enterococcus faecium* (Muck 2010). Zheng et al. (2017) found alfalfa silage treated with *L. plantarum* to achieve a

lower pH, have a higher lactic acid content, and also prevented a clostridial fermentation which occurred in the untreated silage.

Homofermentative lactic acid species are responsible for dominating the silage fermentation by rapidly producing lactic acid and minimizing the growth of undesirable microorganisms, while heterofermentative species are applied for aerobic stability (Muck 2010). *Lactobacillus buchneri* (the most common heterofermentative species) ferment a moderate amount of lactic acid to acetic acid which inhibits yeast and mould growth, contributing to the aerobic stability of silage. According to Kung et al. (2003), research on the aerobic stability of forage crops has concentrated on corn silage and high-moisture corn, but alfalfa silage can experience aerobic deterioration and makes up a large portion of forage fed to dairy cows. Kung et al. (2003) were the first to evaluate the effects of *L. buchneri* 40788 on the fermentation and aerobic stability of alfalfa silage. After 56 days of ensiling, silage treated with *L. bacillus* 40788 had a higher pH, higher concentrations of acetic acid, and lower lactic acid concentrations compared to untreated silage, but had improved aerobic stability due to its production of acetic acid which has anti-fungal properties. Research by Filya et al. (2007) studied the effect of 14 microbial inoculants on the fermentation and nutritive value of alfalfa silage. The inoculants used in this study had a generally positive effect on alfalfa silage characteristics through a lowered pH as well as a shift toward lactic acid with homofermentative LAB, and toward acetic acid with heterofermentative LAB (*L. buchneri*). Wambacq et al. (2013) reported the inoculation of alfalfa-ryegrass with *L. buchneri* to improve aerobic stability as treated silage had significantly fewer mould spores than untreated silage.

Strains of homofermentative and heterofermentative LAB have been combined to evaluate their effects on silage quality. The purpose of these combination inoculants is to take advantage of both types of inoculants to have an initial fermentation controlled by homofermentative strains of LAB resulting in good DM recovery, followed by a later fermentation of a moderate amount of lactic acid to acetic acid performed by *L. buchneri* supporting improved aerobic stability (Muck 2010). Zhang et al. (2009) studied the effect of *L. buchneri* alone or in combination with *L. plantarum* on the fermentation, aerobic stability and bacteria diversity of alfalfa silage. The combination of the heterofermentative and homofermentative LAB resulted in more acetic acid and fewer yeasts and overall was more beneficial than the non-combination treatment because it led to a lower pH and fewer mould spores after 90 days of ensiling. According to Ogunade et al. (2018), proper silage management is necessary to lower mycotoxin contamination of dairy cow feeds, and microbial inoculants can minimize mycotoxin contamination levels. Copani et al. (2018) reported a combination inoculant to reduce concentrations of mycotoxins produced by *Penicillium* spp.

3.1.4 Legume Content

Dewhurst et al. (2003) reported legumes to be more challenging to ensile than grass due to their higher buffering capacity and lower water-soluble carbohydrate content, but all silages were well-fermented. Xue et al. (2020) observed that an increase in alfalfa proportion in mixtures resulted in an increase in pH and a decrease in lactic acid. According to Bijelić et al. (2015), fermentable characteristics of silage can be improved with the addition of grasses.

Xue et al. (2020) reported ammonia to increase with increasing alfalfa proportion in silage mixtures. Papadopoulos and McKersie (1983) reported alfalfa to undergo the highest degree of proteolysis in a comparison of six forage species commonly used in Eastern Canada including alfalfa, red clover (*Trifolium pratense*, birdsfoot trefoil (*Lotus corniculatus* L.), orchardgrass (*Dactylis glomerata* L.) bromegrass (*Bromus inermis* Leyss.) and timothy.

3.1.5 Harvest Date

DM yield of alfalfa increases with maturity, but quality decreases (Cassida et al. 2000). The stem proportion of alfalfa will increase with maturity resulting in a higher fiber concentration and lower fiber digestibility (Lamb et al. 2012). Lignin increases with plant maturity (Moore and Jung 2001) and is a measurement of the undigestible plant component and reduces digestibility (Ball et al. 2001). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) both measure the fiber content of plants; NDF measures the indigestible and slowly digestible components of plant cells walls (cellulose, hemicellulose, lignin and ash), while ADF measures cellulose, lignin and silica (Ball et al. 2001). Cassida et al. (2000) reported NDF and ADF to increase with maturation and crude protein (CP) to decrease.

Delaying the harvest date may influence mycotoxin production; increasing forage maturity causes the tissues of forage to senesce and also causes an increased time frame for infection by filamentous fungi, increasing the opportunity for pre-storage mycotoxin production (McElhinney et al. 2015). Wilkinson and Davies (2013) speculated that crops that are mature with seed heads at advanced maturity may have higher yeast and mould populations on the crop at harvest.

3.1.6 Mechanical Conditioners

Forage harvesting may involve cutting, conditioning, field drying, raking and storage, during which losses of quantity and quality occur (Greenlees et al. 2000). Minimizing losses is crucial to maximizing quality and nutritive value (Greenlees et al. 2000). Forage has 75 to 80% moisture when it is cut in the field and must be dried down to the appropriate moisture content for the storage system (Undersander and Saxe 2013). Plants continue to respire after being cut resulting in the oxidation of organic matter and losses in dry matter (Rotz and Sprott 1984). Loss also occurs due to shatter loss (leaf loss), which is when leaves are stripped off the plant (Rotz and Sprott 1984).

Mechanical conditioning devices have been developed to reduce drying time by promoting moisture loss during wilting, which is achieved in a variety of ways depending on the type of conditioner; for example, roller conditioners crush or crimp the stems, while flail mowers cut the crop (Rotz and Sprott 1984). Leaf losses during harvest may result in only 75% of an alfalfa crop being available for animal consumption; due to leaves being higher in CP and lower in fiber than stem material, the nutritional quality of the alfalfa declines due to increases in fiber concentrations and lower protein (Buckmaster et al. 1990; Rotz and Muck 1994).

3.1.7 Objective and Hypotheses

The objective of this chapter was to evaluate the relationships between silage management and quality parameters identified using factor analysis. The following hypotheses were developed based on the literature review above.

1) Wrapped bales will undergo less fermentation than forage ensiled in bunkers and towers because the forage is not chopped, causing a low population of LAB and therefore a less dramatic drop in pH.

2) Bales will have greater abundance of fungal contaminants than forage ensiled in bunkers and towers because of their more aerobic environment, lower density and higher pH.

3) Silage that has higher DM will undergo more heating and will be more at risk for MPA contamination than silage that has a lower DM.

4) Heat damaged proteins (measured by ADF-CP) will be higher in bales than forage ensiled in bunkers and towers due to their more aerobic environment.

5) The use of an inoculant will increase lactic acid concentrations, lower pH and decrease the incidence of mycotoxin production.

6) Silage with a higher legume content will have a higher buffering capacity resulting in a greater resistance to a lowered pH, potentially resulting in more fungal contaminants and perhaps increased mycotoxin incidence. Samples with higher legume content will also have higher ammonia.

7) First cut forage that was harvested later will have lower CP, and higher lignin, NDF and ADF. It may also result in silage with lower lactic acid and higher pH and potentially higher MPA incidence.

8) Forage that has been mowed with a conditioner will have higher leaf loss resulting in higher NDF, ADF, lignin, and lower CP than forage that has been mowed without a conditioner.

3.2 Materials and Methods

3.2.1 Nutritional Analyses

Silage samples were collected (Chapter 2) and used to perform further chemical analyses to determine their quality. Samples were sent for near-infrared reflectance (NIR) at Cumberland Valley Analytical Services in Waynesboro, Pennsylvania to evaluate the nutritional quality of the silage. Dr. Carole Lafrenière and colleagues at the UQAT performed wet chemistry analyses to measure the quality of the silage, and Dr. Kees Plaizier and colleagues at the University of Manitoba performed feed analyses.

The following wet chemistry procedures were performed by Lafrenière (personal communication) following standard protocols from the Association of Official Analytical Chemists (AOAC) (1990). Samples were kept frozen until their preparation for extraction. Volatile fatty acids (acetic acid, propionic acid, i- and n-butyric acid, i-and n-valeric acid), alcohols (ethanol, 1- propanol, i-propanol, methanol, 1,2-propanediol, 1,2-butanediol) acetone, pH, ammonia and lactic acid were determined in silage extracts. These extracts were prepared by adding 300 g demineralized water to 30 g of silage and homogenizing for 5 min in a laboratory blender (Stomacher 400, Seward Medical, London, UK). Silage extracts were then sieved through layers of cheese cloth. Residual liquid extracts were centrifuged at 18 000 rcf in a refrigerated centrifuge (4°C) for 10 min. Supernatant was divided into 5 different aliquots and kept frozen until further analysis. Each analysis was performed in duplicate except pH.

Ammonia was determined on 20 mL aliquots by distillation with MgO (AOAC 920.03-1920) on an automated Kjeltec 1030 (Foss, Eden Prairie, MN, USA). Lactic acid concentration was determined using the method of Barker and Summerson (1941). The pH was measured with a pH meter (Accumet AB15, Fisher Scientific, Toronto, ON,

Canada) on an aliquot of 25 mL. Volatile fatty acids, alcohols and acetone were determined by gas chromatography using an Agilent 7890A/5975C (Mississauga, ON, Canada) chromatograph equipped with a 60-m DB-WAX capillary column (i.d., 0.25 mm; film thickness, 0.25 μm; Agilent J&W 122-7063UI), a 5 m- guard column Hydroguard FS, (i.d., 0.25 mm; Restek, 20429) and a mass spectrometer detector. Three internal standards were used for the different categories of components. For diols, 1,3-propanediol was used as internal standard. For fatty acids, 2-ethylbutanoic acid was used and for all the others 1-butanol was used. At the time of the sample injection, the column temperature was 50°C for 2 min, then ramped at 15°C min^{-1} until 80°C and maintained for 2 min. Then, temperature was ramped at 120°C min^{-1} to reach 235°C. This temperature was maintained for 5.7 min. At the end, the apparatus was purged for 2 min at 225°C. Inlet, detector and quadrupole temperatures were 250°C, 230°C and 150°C, respectively. The split ratio was 10:1. The flow rate for helium carrier gas was 1.5 mL min^{-1}. Each peak was identified using a commercial database (NIST2008), confirmed and quantified with ACS standards certified over 99%.

To determine the DM of each silage sample, an extraction of water with anhydrous methanol (Methyl alcohol Chromar, VWR, Mississauga, ON) was done according to Petit et al. (1997), using 20 g of silage and 150 mL of anhydrous methanol. The water content was determined by gas chromatography, using an Agilent 7890A/5975C (Mississauga, ON, Canada) chromatograph equipped with a 60-m DB-WAX capillary column (i.d., 0.25 mm; film thickness, 0.25 μm; Agilent J&W 122-7063UI), a 5 m- guard column Hydroguard FS, (i.d., 0.25 mm; Restek, 20429) and a mass spectrometer detector. At the time of the sample injection the column, the

temperature was 70°C for 1 min, then ramped at 70°C min^{-1} until 240°C and maintained for 3 min. At the end, the apparatus was purged for 8 min, at 240°C. Inlet, detector and quadrupole temperatures were 250°C, 230°C and 150°C, respectively. The split ratio was 100:1. The flow rate for helium carrier gas was 1.5 mL min^{-1}.

Information provided from NIR analyses allowed the percent legume in the silage samples to be calculated using the second equation from Allard et al. (2001): % legume = 119.265 CP/ADF + 352.415 ADF/NDF – 238.685, where CP is crude protein, ADF is acid detergent fiber, and NDF is neutral detergent fiber.

The following procedure to evaluate feed quality of the silage samples was provided by Plaizier (personal communication). Analytical DM for pooled samples for each stage was determined (method 934.01; AOAC 1990). All feed samples were analyzed for NDF according to Van Soest et al., (1991) using α-amylase (Sigma No. A3306, Sigma Chemical Co., St. Louis, MO), and sodium sulfite, and corrected for ash concentration using an ANKOM 200 Fiber Analyzer (ANKOM Technology, Fairport, NY. Feed samples were also analyzed for crude protein using the $CuSO_4/TiO_2$ mixed catalyst Kjeldahl procedure (method 988.05; AOAC 1990). Analyses of ADF, ether extract by extraction, and ash in a furnace oven of feed samples were conducted using AOAC method 973.18 (AOAC, 1990), AOAC method 920.39 (AOAC 1990) and AOAC method 923.03 (AOAC 2005), respectively. Calcium, P, K, Mg, and Na in feed samples were measured by inductively coupled plasma emission spectroscopy (method 968.08; AOAC 1990) using an Atom Scan 25 Plasma Spectrometer (Thermo Jarrell Ash Corp., Grand Junction, CO) after acid digestion.

3.2.2 Survey Questionnaire

In 2019, a survey questionnaire regarding a wide range of silage management practices was developed by the University of Manitoba with input from other researchers involved in the national study as well as dairy producers and industry professionals. This survey was further improved by Christina Waddy of Narrative Research in Moncton, New Brunswick. The survey was trialed with producers on a small scale throughout the process. Survey questionnaires were hand delivered or emailed to farms in late 2019 and early 2020 and completed by extension personnel, researchers or graduate students. Producers were told that it was a voluntary survey, but that the survey would help to explain the quality of the silage. The survey questionnaire included questions on botanical composition of forage stands, age of stands, the use of inoculants, equipment used, silage management and storage management. A copy of the survey is included in the appendix (A4).

3.2.3 Statistical Analyses

The information in this chapter is based on data from the 2018 silage samples. Factor analysis using the PROC FACTOR procedure in SAS software (version 9.4; SAS Institute Inc., Cary NC) was used to identify variables obtained from NIR, digestibility data, wet chemistry and survey data that had highest loading values in a 3-factor factor analysis. Analysis of variance (ANOVA) was conducted using PROC GLIMMIX in SAS with these variables as well as legume content, DM and harvest date used as covariates because they were continuous variables. Means were compared using Fisher's protected LSD test at significance level of α=0.05 where *Pr*>F was less than or equal to 0.10. Pearson's correlation analysis was run on to assess linear relationships between dependent variables and covariates with significance determined at α=0.05. A

comparison of feature counts obtained from bioinformatic analyses in Chapter 2 was performed using one-way ANOVA conducted in Minitab Statistical Software (Version 19.2020.1.0. 2020. Minitab Inc., State College, PA) with significance determined at α=0.05.

3.3 Results and Discussion

3.3.1 Factor Analysis

Table 3.1. Factors identified in factor analysis using SAS software and factor loading in a 3-factor analysis.

Factor 1	Factor 2	Factor 3
Crude protein (%DM)	Ammonia (%DM)	Acetic Acid (%DM)
ADF-CP (%CP)	Ash (%DM)	n-Butyric Acid (%DM)
Lignin (%DM)		Lactic Acid (%DM)
NDF30 (%DM)		
Legume content (%)		
pH		
MPA ($\mu g\ kg^{-1}$)		
ADF (%DM)		
NFC (%DM)		
TDN (%DM)		

CP= crude protein, ADF-CP= acid detergent fiber crude protein, NDF30= neutral detergent fiber at 30 h, MPA= mycophenolic acid, ADF= acid detergent fiber, NFC= nonfibrous carbohydrates, TDN= total digestible nutrients, DM=dry matter

The use of factor analysis in SAS software as a means of data reduction identified the factors presented in Table 3.1. The information obtained from NIR, digestibility, wet chemistry, and the surveys generated a large amount of data. Factor analysis was used as a means of data reduction to explain the largest amount of variation with fewer variables. Factor analysis was used to identify which dependent variables to evaluate and subsequent analyses were conducted based on this information.

Table 3.2. Simple statistics obtained for variables identified in factor analysis with the addition of covariates DM and harvest date, and the method used to obtain the data.

Variable	Method	N	Mean	S.D.	Median	Min	Max
Lactic Acid (%DM)	WC	76	3.87	2.61	3.62	0	11.44
Acetic Acid (%DM)	WC	76	1.69	1.01	1.52	0.18	5.17
n-Butyric Acid (%DM)	WC	76	0.14	0.25	0.05	0	1.27
pH	WC	76	4.72	0.72	4.56	3.76	8.93
Ammonia (%DM)	WC	76	0.19	0.10	0.18	0.0	0.63
CP (%DM)	NIR	65	16.2	3.14	15.90	8.50	22.70
ADF-CP (%CP)	NIR	65	8.86	3.78	8.20	4.20	28.30
ADF (%DM)	NIR	74	33.38	4.55	32.89	25.02	49.33
NDF30 (%DM)	NIR	65	30.43	6.63	30.30	16.50	47.70
Lignin (%DM)	NIR	65	5.70	1.51	5.37	3.20	11.20
NFC (%DM)	ANKOM	74	24.65	4.89	24.99	12.11	33.76
TDN (%DM)	ANKOM	74	62.98	4.87	63.50	45.93	71.91
Ash (%DM)	WC	74	8.90	1.58	8.78	5.64	13.37
MPA ($\mu g\ kg^{-1}$)	LC-MS/MS	91	0.14	0.55	0	0	3.48
Legume content (%)	NIR	65	61.82	28.27	61.09	12.70	100
DM (%)	NIR	65	42.74	9.42	41.80	26.40	79.60
Harvest Date	NA	46	160	9.54	161	145	184

CP= crude protein, ADF-CP= acid detergent fiber crude protein, ADF= acid detergent fiber, NDF= neutral detergent fiber at 30 h, NFC= nonfibrous carbohydrates, TDN= total digestible nutrients, MPA= mycophenolic acid, DM= dry matter, Harvest date= Julian date, WC= wet chemistry, NIR= near infrared reflectance, LC-MS/MS= liquid chromatography tandem mass spectrometry immunosorbent assay, S.D. = standard deviation, Min= minimum, Max= maximum

Table 3.2 provides the simple statistics for the variables obtained in factor analysis. In some cases, there was not enough silage material to perform all the tests. A complete list of the variables measured by each test for silage samples in 2018 can be found in Table A2 of the appendix. Additional information regarding tests performed, mycotoxins detected, and other sample specific information is provided in Table A3 of the appendix.

Table 3.3. P-values obtained using ANOVA to evaluate the effects of management practices on the factors identified in factor analysis.

Variable	Storage Type	Conditioner	Inoculant	Number of Harvests	NMP	Legume Content	DM	Harvest Date
CP (%DM)	**0.0013**	0.42	**0.0522**	0.60	0.10	**<0.001**	0.98	0.74
ADF-CP (%CP)	**0.0011**	0.57	0.16	0.84	0.15	**0.0721**	**0.0428**	0.10
Lignin (%DM)	**0.0179**	**0.0166**	**0.0652**	0.11	0.49	0.71	0.90	0.54
NDF30 (%DM)	0.48	0.34	0.23	0.54	0.70	**<0.001**	**0.0264**	0.76
pH	**0.0015**	0.20	**0.0139**	0.71	0.90	0.88	**0.0130**	0.36
Lactic Acid (%DM)	**0.0874**	0.69	**0.0255**	0.34	0.62	**0.0155**	**0.0030**	**0.0217**
MPA ($\mu g\ kg^{-1}$)	0.41	0.14	0.50	0.41	0.97	0.33	0.36	0.29
ADF (%DM)	0.16	0.20	0.70	0.21	0.83	**0.0239**	0.11	0.21
NFC (%DM)	0.94	0.29	0.60	0.46	0.33	**0.003**	0.19	0.95
TDN (%DM)	0.16	0.20	0.69	0.21	0.83	**0.0239**	0.11	0.21
Ammonia (%DM)	0.79	0.17	0.22	0.71	0.15	**0.0818**	0.23	0.95
Ash (%DM)	**0.0349**	0.22	0.24	0.43	0.86	**<0.001**	0.58	0.57
Acetic Acid (%DM)	**0.0033**	0.55	0.78	0.43	0.22	0.29	**0.0163**	**0.0679**
n-Butyric Acid (%DM)	**0.0458**	**0.0048**	**0.0862**	0.26	**0.0267**	0.97	0.91	**0.0040**

CP= crude protein, ADF-CP= acid detergent fiber crude protein, NDF= neutral detergent fiber at 30 h, MPA= mycophenolic acid, ADF= acid detergent fiber, NFC= nonfibrous carbohydrates, TDN= total digestible nutrients, DM= dry matter, Number of harvests= how many harvests producers took on their alfalfa-grass land, NMP= nutrient management plan
P-values <0.05 are considered significant and 0.05>p-values <0.10 marginally significant.

3.3.2 Effect of Storage Type on Silage Quality Silo

Table 3.4. Means of variables determined to be significantly affected by storage type using ANOVA.

Variable	Bale	SE	Bunker	SE	Tower	SE
CP (%DM)	16.9*a*	0.5177	16.3*a*	0.5078	14.8*b*	0.4613
ADF-CP (%CP)	5.83*b*	0.8539	9.48*a*	0.8376	9.56*a*	0.7608
Lignin (%DM)	4.41*b*	0.3364	5.09*ab*	0.3300	5.51*a*	0.2998
pH	4.86*a*	0.1413	4.57*b*	0.1341	4.23*b*	0.1224
Lactic acid (%DM)	3.46*b*	0.8040	4.64*ab*	0.7631	5.45*a*	0.6964
Ash (%DM)	8.65*a*	0.4428	8.59*a*	0.4920	7.56*b*	0.3882
Acetic Acid (%DM)	0.58*b*	0.3861	2.36*a*	0.3665	1.91*a*	0.3345
n-Butyric Acid (%DM)	*0b*	0.08961	0.21*b*	0.08505	0.23*a*	0.07762

SE= standard error, CP= crude protein, ADF-CP= acid detergent fiber crude protein, DM= dry matter
Means with the same letters within rows are not significantly different according to Fisher's protected LSD test at α= 0.05 where *Pr*>F was less than or equal to 0.10.

Table 3.5. P-values and correlation coefficients obtained from Pearson's correlation of covariates with factors identified in factor analysis.

Variable	Legume Content			DM			Harvest Date		
	r	p-value	n	r	p-value	n	r	p-value	n
CP (%DM)	0.86	**<.0001**	65	-0.04	0.78	65	-0.40	**0.0139**	38
ADF-CP (%CP)	-0.33	**0.0075**	65	0.06	0.65	65	0.39	**0.0159**	38
Lignin (%DM)	0.20	0.12	65	0.07	0.56	65	0.22	0.19	38
NDF30 (%DM)	-0.89	**<.0001**	65	-0.12	0.33	65	0.19	0.25	38
pH	-0.11	0.40	62	0.25	**0.048**	62	0.23	0.16	39
Lactic Acid (%DM)	0.32	**0.0124**	62	-0.59	**<.0001**	62	-0.03	0.83	39
MPA ($\mu g\ kg^{-1}$)	-0.12	0.34	65	-0.07	0.56	65	0.10	0.49	46
ADF (%DM)	-0.17	0.19	64	-0.14	0.28	64	0.14	0.42	37
NFC (%DM)	0.48	**<.0001**	64	0.04	0.75	64	-0.28	**0.0873**	37
TDN (%DM)	0.17	0.19	64	0.14	0.28	64	-0.14	0.42	37
Ammonia (%DM)	0.43	**0.0005**	62	-0.41	**0.0011**	62	-0.05	0.78	39
Ash (%DM)	0.62	**<.0001**	64	-0.08	0.52	64	-0.16	0.35	37
Acetic Acid (%DM)	-0.03	0.80	62	-0.49	**<.0001**	62	0.10	0.55	39
n-Butyric Acid (%DM)	-0.24	**0.0634**	62	-0.23	**0.0689**	62	0.44	**0.0048**	39

CP= crude protein, ADF-CP= acid detergent fiber crude protein, NDF= neutral detergent fiber at 30 h, MPA= mycophenolic acid, ADF= acid detergent fiber, NFC= nonfibrous carbohydrates, TDN= total digestible nutrients, DM= dry matter
values <0.05 are considered significant and 0.05>p-values <0.10 marginally significant.

Storage type affected the CP, ADF-CP, lignin and ash, and the chemical characteristics lactic acid, acetic acid, n-butyric acid and pH (Table 3.3). Bales underwent

a limited fermentation as indicated by higher pH levels (Table 3.4), which Goering and Van Soest (1972) associated with higher heat damage, but bales had statistically lower ADF-CP than bunkers and towers (Table 3.4). Higher ADF-CP in bales was unexpected as bales typically cannot achieve the higher densities that bunker silos do and oxygen concentrations are higher in bales which can delay fermentation (Bernardes et al. 2018). Bales may have had lower heat-damaged protein because they were wrapped, and oxygen excluded in a relatively short time frame compared to when towers and bunkers were covered. All the bales in this study were wrapped within 24 h, whereas some tower silos were only covered after 14 days. Another possible explanation for lower ADF-CP in bales could be due to lower lignin in bales (Table 3.4). ADF-CP and harvest date were positively correlated (Table 3.5) and since lignin is an indicator of maturity, perhaps producers that are harvesting earlier are also implementing ensiling techniques to reduce heating. DM of silage did have an effect on ADF-CP, but it did not have a significant effect on MPA (Table 3.3).

Ash is a measure of the total mineral content of the forage with values >10% for grasses and 14% for legumes commonly reflecting soil contamination (Ball et al. 2001). Soil is a source of *Clostridia* (Pahlow et al. 2003) which can form butyric acid and degrade proteins to ammonia (Danner et al. 2003). Storage type was found to significantly affect ash content (Table 3.3). There were no differences in ash content between bale and bunker silage, which was previously reported by McElhinney et al. (2015), while tower silos had lower ash content than both other types (Table 3.4). This could be because there is less soil contamination when ensiling forage in a tower. Most bales are picked up from the field and transported back to the farm to be wrapped. All of

the bales in this study were wrapped on the ground allowing for further potential soil contamination. Soil contamination in bunkers can occur if the packing equipment is rolling back into soil while packing the bunker.

In this study, bales had higher pH and lower lactic acid than bunkers and towers (Table 3.4) as hypothesized. It is presumed this less complete fermentation in bales could be attributed to the forage not being chopped, resulting in less plant sugars diffusing out of the plant and restricting LAB growth as suggested by Muck (1989). Bales had lower acetic acid levels than bunkers and towers (Table 3.4) suggesting that bales may be more prone to aerobic deterioration. In this study, pH, lactic acid and acetic acid were all affected by DM (Table 3.3). pH and DM were positively correlated while lactic acid and DM were negatively correlated (Table 3.5). Butyric acid levels were higher in tower silos (Table 3.4) perhaps due to difficulties wilting the forage to a sufficient DM, but butyric acid levels were not significantly affected by DM (Table 3.3).

The second hypothesis of this study was that bales would have greater abundance of fungal contaminants due to their more aerobic environment, lower density and higher pH. A comparison of features counts obtained from bioinformatic analyses in Chapter 2 for the different storage types yielded no significant differences. Perhaps this is because bales were wrapped quickly and had lower heat-damaged protein. Bales may have been more airtight in comparison to other storage types; bunker silos typically have an exposed face at feed out, and tower silos are prone to oxygen ingress because they are not completely airtight.

MPA was detected in six bale and two bunker samples. There were no mycotoxins above detectable limits in tower silos. Storage type did not significantly

affect MPA (Table 3.3). The number of MPA-positive samples was 20% in bales and 6.7% in bunkers, while McElhinney et al. (2015) reported 8.9% in bales and 2.1% in bunkers in year one and 1.8% in bales and 0% in bunkers in year two. The presence of MPA could be due to formation in the field or in the silo. Samples that tested positive for MPA, where sampling took place very shortly after ensiling in MB, included one bunker and two bale samples, indicating MPA formation likely occurred in the field.

3.3.3 Effect of Inoculant on Silage Quality

Table 3.6. Means of variables determined to be significantly affected by the use of an inoculant using ANOVA.

Variable	Inoculant Used	SE	Inoculant not Used	SE
CP (%DM)	16.5*a*	0.4180	15.6*b*	0.4271
Lignin (%DM)	4.71*b*	0.2716	5.30*a*	0.2775
pH	4.35*b*	0.1102	4.68*a*	0.1151
Lactic acid (%DM)	5.36*a*	0.6272	3.67*b*	0.6551
n-Butyric Acid (%DM)	0.07*b*	0.06991	0.21*a*	0.07302

SE= standard error, CP= crude protein, DM= dry matter
Means with the same letters within rows are not significantly different according to Fisher's protected LSD test at α= 0.05 where *Pr*>F was less than or equal to 0.10.

The use of an inoculant resulted in silage with a lower pH and higher lactic acid (Table 3.6) which is in agreement with the reports above (Kung et al. 2003; Filya et al. 2007; Zheng et al. 2017). Insufficient survey data was obtained to assess the type of inoculant used. Butyric acid levels were higher in silage that was not inoculated but this was only marginally significant (Tables 3.3, 3.6). Although inoculants were successful in increasing lactic acid levels and decreasing pH, they did not significantly decrease the presence of MPA.

Silage that was inoculated also had marginally higher CP and marginally lower lignin (Table 3.6). This could be an indication that producers that are inoculating are also

harvesting their forage sooner as evidenced by the negative correlation between harvest date and CP (Table 3.5), but ANOVA determined lignin and CP were not significantly affected by harvest date (Table 3.3). The higher CP may also be an indication that producers that are growing higher legume content forage are more likely to inoculate and encourage an optimal fermentation.

3.3.4 Effect of Legume Content on Silage Quality

Legume content significantly affected lactic acid concentrations, but had no effect on pH or acetic acid (Table 3.3). A positive correlation between legume content and lactic acid (Table 3.5) may suggest that the same producers that had higher legume content in their forage stands were more likely to use inoculants and management practices to aid an ideal fermentation. There was a marginally significant negative correlation between legume content and butyric acid (Table 3.5). This may also be due to management practices implemented by producers that would have reduced the concentration of *Clostridia* responsible for producing this acid such as a rapid drop in pH (Muck 2010). Legume content had a marginally significant effect on ADF-CP (Table 3.3) and these two variables were negatively correlated (Table 3.5) which could be due to management; producers that grew higher legume forage stands may have implemented better ensiling techniques as discussed above. It was hypothesized that legume content would affect MPA, but this did not occur (Table 3.3), potentially because legume content did not have a negative effect on lactic acid and pH; samples with higher legume content actually had higher lactic acid concentrations, likely due to the use of an inoculant. Ash content increased with legume content (Table 3.5) likely because legumes are naturally

higher in minerals. Ammonia levels were marginally significantly affected by legume content (Table 3.3) with a positive correlation found (Table 3.5) as hypothesized.

3.3.5 Effect of Harvest Date on Silage Quality

It was hypothesized that first cut forage that was harvested later will have higher CP, and lower lignin, NDF and ADF. It may also result in silage with lower lactic acid and higher pH and potentially higher MPA incidence. Harvest date significantly affected lactic acid, acetic acid and butyric acid concentrations (Table 3.3). Later harvest dates were associated with higher n-butyric acid concentrations (Table 3.5) which could be due to the forage being ensiled at a low DM. MPA was not impacted by harvest date. NDF, ADF, and lignin are influenced by plant maturity, but harvest date did not have a statistically significant effect on them (Table 3.3). ANOVA did not report CP to be significantly affected by harvest date (Table 3.3), but there was a significant negative correlation found between these two variables (Table 3.5). Lack of differences found in fiber components could be because this study collected data from across Canada from different regions with differing harvest dates; an early harvest date in Atlantic Canada may be considered a later harvest date in parts of Quebec and Ontario.

3.3.6 Effect of Conditioner on Silage Quality

Table 3.7. Means of variables determined to be significantly affected by the use of a conditioner using ANOVA.

Variable	Conditioner Used	SE	Conditioner not Used	SE
Lignin (%DM)	5.43*a*	0.2889	4.58*b*	0.2767
Butyric Acid (%DM)	0.01*b*	0.07559	0.27*a*	0.07143

SE= standard error, DM= dry matter
Means with the same letters within rows are not significantly different according to Fisher's protected LSD test at $\alpha = 0.05$ where *Pr*>F was less than or equal to 0.10.

The use of a conditioner resulted in forage with higher lignin (Table 3.7) likely attributed to leaf loss. While forage cut with a conditioner may have undergone higher leaf loss as hypothesized, NDF, ADF or CP were not impacted. Forage cut without a conditioner had higher butyric acid levels (Table 3.7), suggesting that the forage was not dried sufficiently.

3.4 Conclusion

Chemical characteristics of the silage showed bales underwent a less complete fermentation than bunkers and towers as evidenced by their higher pH and lower lactic acid. Bales may also be less aerobically stable due to lower acetic acid levels. Despite bales undergoing a less desirable fermentation, they did not have higher feature counts than bunkers and towers. Higher levels of ash in bunkers and bales than towers could be attributed to an increased risk of soil contamination when bales are transported from the field to the farm to be wrapped, or the manner in which forage in bunkers are packed. Bales did not have higher heat damaged protein as hypothesized; they actually had lower ADF-CP than bunkers and towers, perhaps due to good management practices including harvesting forage at an ideal maturity and wrapping quickly.

Inoculant was successful in lowering the pH and increasing lactic acid concentrations in silage but did not affect MPA production. Legume content had no effect on MPA. Silage with higher legume content did not negatively affect chemical characteristics as hypothesized; lactic acid concentrations were actually positively correlated with legume content, perhaps because producers that were growing higher levels of alfalfa in their forage stands were also using an inoculant and implementing

good ensiling techniques. Legume content and ammonia levels were positively correlated.

Harvest date did not influence fiber components or MPA, but did affect lactic acid, acetic acid and n-butyric acid concentrations. Forage that was not conditioned had lower lignin but had higher n-butyric acid levels suggesting the forage was not wilted sufficiently.

Chapter 4: Conclusion

4.1 Chapter 2 Conclusion and Future Research

Chapter 2 sought to address the first objective of this research: to detect and identify fungal species and populations in silage, and to determine whether mycotoxin-associated species are present in silage. This metagenomic approach using NGS technology to investigate fungal communities in alfalfa-grass silage has not been attempted before, especially to this extent using a nationwide on-farm research approach. The most concerning yeasts in the 20 most abundant yeasts were *Pichia anomala* and *Pichia kudriavzevii* because of their fermentative and lactate-assimilating abilities and association with aerobic deterioration in silage. The moulds of most concern in the 20 most abundant ASVs were *Monascus* sp., and *Penicillium* sp. due to their mycotoxin production capabilities.

Fungal presence and abundance differed across Canada, perhaps due to climatic and management factors, but more likely due to sampling time. Samples in Western Canada were sampled soon after ensiling in the summer months, while samples east of Western Canada were sampled in the winter months. Many of the fungi identified in Western Canada are reported to decline with ensiling time. Some fungi in this study have been documented in other studies with a similar temperate climate such as *Penicillium* spp., *Pichia anomala*, *Pichia fermentans*, but *Geotrichum* spp. and *Schizophyllum commune* were not observed, perhaps due to climatic and management factors. Future studies should sample silage at relatively the same time to be able to facilitate regional comparisons.

This research indicated that there was very little mycotoxin contamination in silage and only low levels of mycotoxin-associated species were detected in metagenomic sequencing. The low incidence of mycotoxins in this study (ZEA detected in 1% of samples and MPA in 11%), could be due to a variety of factors; type of crop, sample collection time, harvest time, mycotoxin degradation. Although mycotoxin concentrations and incidence were lower than other areas of the world, research is lacking on the chronic and systemic effects of these mycotoxins on ruminant health. There does not appear to be any research conducted on the long-term health risks for dairy cattle consuming low levels of mycotoxins such as MPA.

Only low levels of mycotoxin-associated species were detected in metagenomic sequencing from first cut silage, but this may change with cutting date. Choosing to sample first cut forage was an appropriate decision as it typically produces most of the yield, has the best quality and is arguably the most important harvest. However, it would be interesting to sample subsequent cuts, especially those cut in July and August to assess if mycotoxin incidence and concentration is higher, as this is when small grains are usually infected due to climatic factors such as temperature, precipitation and humidity.

Perhaps silage should be monitored for mycotoxin contamination sooner after harvest, especially if mycotoxins degraded before sampling time in the winter, to asses risk to animal health. More silage samples could be collected from farms, particularly those that had detectable mycotoxin presence to evaluate if it is an on-going concern. Future research may focus on correlations between chemical parameters and key species of fungi. Producers could be alerted if their silage samples fall into certain categories of

concern conducive to yeasts associated with aerobic deterioration and mycotoxin-associated species.

While the package selected for mycotoxin detection was broad, it did not include testing for citrinin. It is recommended that alfalfa-grass silage samples in Canada be tested for citrinin as the most abundant feature in this study belonged to the genus *Monascus*. While this feature could not be identified to the species level, the two possible species, *M. ruber* and *M. purpureus*, are both capable of citrinin production. Another possible mycotoxin to test for is deoxynivalenol-3-β-D-glucoside which is produced from the enzymatic conjugation of glucose to DON (Berthiller et al. 2015).

This research extracted DNA from silage, but future research may focus on extracting RNA which requires much more stringent storage conditions but will broaden our knowledge of what is occurring in the silage, particularly in the time frame immediately prior to sampling. qPCR could be used to target polyketide synthases genes which are responsible for the production of polyketides, a large group of secondary metabolites. qPCR primers could be developed to investigate genes of interest in the polyketide system, to assess the possibility of mycotoxin production.

4.2 Chapter 3 Conclusion and Future Research

The objective of Chapter 3 was to evaluate the relationships between silage management and quality parameters identified using factor analysis from a large dataset containing information on NIR, digestibility, wet chemistry, and survey data. Factor analysis was used to reduce this database into fewer factors that explained a large amount of the variation.

Inoculant improved silage chemical characteristics by lowering the pH and increasing lactic acid concentrations but MPA was not affected. The use of an inoculant likely counteracted the challenges of ensiling legume forages through the production of lactic acid, lowering the pH. Insufficient information was obtained from survey data to assess inoculant type (homofermentative LAB, heterofermentative LAB, or combination) which future research should focus on, particularly the effectiveness of combination inoculants on improving initial fermentation followed by increased aerobic stability using an on-farm approach.

Bales underwent a less complete fermentation than silage in bunkers and bales as indicated by their lower lactic acid levels and higher pH than bunkers and towers and may have been more prone to aerobic deterioration due to lower acetic acid levels, but there was no difference observed in the relative abundance of most fungal ASVs. Bales had lower heat damaged protein as measered by ADF-CP than bunkers and towers, perhaps because bales were wrapped relatively quickly, but more likely due to lower lignin levels, suggesting that producers that were harvesting early were also implementing good ensiling techniques.

Dairy producers storing forage in tower silos should evaluate their silo for structural deterioration and its susceptibility to oxygen ingress. This research showed that tower silos are not airtight as indicated by yeast activity, and they do not maintain forage quality well. Some silage samples were collected from oxygen limiting silos in 2019. It will be interesting to compare the quality obtained in them compared to conventional concrete stave tower silos. It is a costly recommendation that dairy producers move away from conventional tower silos, but a great deal of work is involved with producing high

quality forage and it must be maintained for the animal. An alternative to adjusting the storage type is applying shock crete to the cracks between staves where oxygen is gaining entry. Inoculants containing oxygen-scavenging LAB may help with oxygen entry, not just for tower silos, but all storage types. Future research may focus on the effectiveness of these inoculants.

Bunker silos appeared to be intermediate in terms of quality preservation. This storage type had a high abundance of the yeast *Pichia kudriavzevii* which is associated with aerobic deterioration, but had a low abundance of other features found in tower and bale samples. Bunker samples had higher heat damaged protein (ADF-CP) than bales likely due to the amount of time taken to fill and cover it. Dairy producers should be filling and sealing their silos as quickly as possible, while packing adequately to remove oxygen, so the anaerobic phase of ensiling can begin.

These research findings will contribute to a larger project and be used to develop management plans that are specific to different types of silage storage systems in different regions of Canada in order to improve cow health and longevity and reduce the cost of milk production. This thesis presented information from only one year (2018) but there is still another year of data on NIR, wet chemistry and feed analyses that will be available soon. An economic analysis of dairy farms across Canada is forthcoming and will further contribute to this research.

REFERENCES

Abdullabekova, D.A., Magomedova, E.S., Magomedov, G.G., Aliverdieva, D.A., and Kachalkin, A.V. 2017. Yeast communities of chestnut soils under vineyards in Dagestan. Eurasian Soil Sci. **50**(12): 1463-1467.

Abraham, E.P. 1945. The effect of mycophenolic acid on the growth of *Staphylococcus aureus* in heart broth. Biochem. J. **39**: 398-404.

Abrams, R., and Bently, M. 1959. Biosynthesis of nucleic acid purines. Biochem. Biophys. Acta **79**: 91-97.

Adesogan, A.T., Krueger, N., Salawu, M.B., Dean, D.B., and Staples, C.R. 2004. The influence of treatment with dual-purpose bacterial inoculants or soluble carbohydrates on the fermentation and aerobic stability of Bermuda grass. J. Dairy Sci. **87**: 3407-3416.

Adesogan, A.T. 2018. Anti-quality factors with forage baleage and grain crop silage. J. Anim. Sci. **96**: 63-64.

Adom, F., Maes, A., Workman, C., Clayton-Nierderman, Z., Thoma, G., and Shonnard, D. 2012. Regional carbon footprint analysis of dairy feeds for milk production in the USA. Int. J. Life Cycle Assess. **17**: 520-534.

Akorli, J., Namaali, P.A., Ametsi, G.W., Egyirifa, R.K., and Pels, N.A.P. 2019. Generational conservation of composition and diversity of field-acquired midgut microbiota in *Anopheles gambiae* (*sensu lato*) during colonization in the laboratory. Parasites and Vectors. **12**(27).

Allard, G., Brégard, A., Gosselin, B., and Pellerin, D. 2001. Proportion de légumineuses dans les fourrages? Des équations pour la calculer! Demi-journée d'information scientifique sur les fourrages. 22-24.

Alonso, V.A., Pereyra, C.M., Keller, L.A.M., Dalcero, A.M., Rosa, C.A.R., Chiacciera, S.M., and Cavaglieri, L.R. 2013. Fungi and mycotoxins in silage: a review. J. Appl. Microbiol. **115**(3): 637-643.

Amigot, S.L., Fulgueira, L., Bottai, H., Basílico, J.C. 2006. New parameters to evaluate forage quality. Postharvest Biol. Technol. **41**(2): 215-224.

Amrouche, T., Mounier, J., Pawtowski, A., Thomas, F., and Picot, A. 2019. Microbiota associated with Dromedary Camel milk from Algerian Sahara. Curr. Microbiol. **77**: 24-31.

Anguita-Maeso, M., Olivares-García, C., Haro, C., Imperial, J., Navas-Cortés, J.A., and Landa, B.B. 2020. Culture-dependent and culture-independent characterization of the olive xylem microbiota: effect of sap extraction methods. Front. Plant Sci. **10**: 1708.

Angulo, M., Reyes-Becerril, M., Cepeda-Palacios, R., Tovar-Ramírez, D., Esteban, M.A., and Angulo, C. 2019. Probiotic effects of marine *Debaryomyces hansenii* CBS 8339 on innate immune and antioxidant parameters in newborn goats. Appl. Microbiol. Biotechnol. **103**: 2339-2352.

Association of Official Analytical Chemists (AOAC). 1990. Official methods of analysis. 15th ed. AOAC, Washington, DC.

Auerbach, H., Oldenburg, E., Weissbach, F. 1998. Incidence of *Penicillium roqueforti* and Roquefortine C in silages. J. Sci. Food Agric. **76**: 565-572.

Ávila, C.L.S., and Carvalho, B.F. 2019. Silage fermentation – updates focusing on the performance of micro-organisms. J. Appl. Microbiol. **128**(4): 966-984.

Ball, D.M., Collins, M., Lacefield, G.D., Martin, N.P., Mertens, D.A., Olson, K.E., Putnam, D.H., Undersand, D.J., and Wolf, M.W. 2001. Understanding Forage Quality. American Farm Bureau Federations Publication 1-01, Park Ridge, IL.

Barker, S.B., and Summerson, W.H. 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. **138**: 535-554.

Bennett, J.W. 1987. Mycotoxins, mycotoxicoses, mycotoxicology and mycopathology. Mycopathlogia **100**:3-5.

Bennett, J.W., and Klich, M. 2003. Mycotoxins. Clin. Microbiol. Rev. **16**(3): 497-516.

Bentley, R. 2000. Mycophenolic acid: A one hundred odysses from antibiotic to immunosuppressant. Chem. Rev. **100**(2000): 3801-3826.

Behjati, S., and Tarpey, P.S. 2013. What is next generation sequencing? Arch. Dis. Child. Educ. Pract. Ed. **98**: 236-238.

Bernard, J.K. 2011. Feed Ingredients | Feed Concentrates: Oilseed and Oilseed Meals. Encyclopedia of Dairy Sciences. 349-355.

Bernardes, T.F., Daniel, J.L.P., Adesogan, A.T., McAllister, T.A., Drouin, P., Nussio, L.G., Huhtanen, P. Tremblay, G.F., Bélanger, G., and Cai, Y. 2018. Silage review: unique challenges of silages made in hot and cold regions. J. Dairy Sci. **101**(5): 4001-4019.

Berthiller, F., Dall'Asta, C., Schuhmacher, R., Lemmens, M., Adam, G., Krska, R. 2005. Masked mycotoxins: determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry. J. Agric. Food Chem. **53**: 3421-3425.

Bijelić, Z., Tomić, Z. Ružić-Muslić, D., Krnjaja, V., Mandić, V., Petričević, M., Caro-Petrović V. 2015. Silage fermentation characteristics of grass-legume mixtures harvested at two different maturity stages. Biotechnol. Anim. Husb. **31**(2): 303-311.

Blakeman, J.P. (ed.) 1981. Microbial ecology of the phylloplane. Academic Press, London.

Blanc, P.J., Loret, M.O., and Goma, G. 1995. Production of citrinin by various species of *Monascus.* Biotechnol. Lett. **17**(3): 291-294.

Blixt, E., Olson, Å., Lindahl, B., Djurle, A., and Yuen, J. 2010. Spatiotemporal variation in the fungal community associated with wheat leaves showing symptoms similar to stagonospora nodorum blotch. Eur. J. Plant Pathol. **126**: 373-386.

Bolysen, E., Rideout, J.R., Dillon, M.R., Bokulich, M.R., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F. et al. 2019. Reproduction, interaction, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. **37**: 852-857.

Boudra, H., and Morgavi, D.P. 2005. Mycotoxin risk evaluation in feeds contaminated by *Aspergillus fumigatus*. Anim. Feed Sci. Technol. **120**(1-2): 113-123.

Boudra, H., and Morgavi, D.P. 2008. Reduction in *Fusarium* toxin levels in corn silage with low dry matter and storage time. J. Agric. Food Chem. **56**: 4523-4528.

Boutigny, A.-L., Gautier, A., Basler, R., Dauthieux, F., Leite, S., Valade, R., Aguayo, J., Ioos, R., and Laval, V. 2019. Metabarcoding targeting the EF1 alpha region to assess *Fusarium* diversity on cereals. PLoS ONE. **14**(1).

Boysen, M.E., Jacobsson, K.-G., and Schnürer, J. 2000. Molecular identification of species from the *Pencillium roqueforti* group associated with spoiled animal feed. Appl. Environ. Microbiol. **66**(4): 1523-1526.

Boysen, M., Skouboe, P., Frisvad, J., and Rossen, L. 1996. Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. Microbiology. **142**: 541-549.

Brakhage, A.A. 2013. Regulation of fungal secondary metabolism. Nat. Rev. Microbio. **11**: 21-32.

Buckmaster, D.R, Rotz, C.A., and J.R Black. 1990. Value of alfalfa losses on dairy farms. Trans. ASAE **33**:351-360.

Callahan, B.J., McMurdie, P.J., and Holmes, S.P. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. ISME J. **11**: 2639-2643.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., and Holmes, S.P. 2016. DADA2: high resolution sample inference from Illumina amplicon data. Nat. Methods. **13**: 581-583.

Caloni, F., Spotti, M., Auerbach, H., Op den Camp, H., Fink Gremmels, J., and Pompa, G., 2000. *In vitro* metabolism of fumonisin B1 by ruminal microflora.Vet. Res. Commun. **24**(6): 379-387.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Gonzalez Peña, A., Goodrich, J.K., Gordon, J.I., et al. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. **7(5)**: 335-336.

Capstaff, N.M., and Miller, A.J. 2018. Improving the yield and nutritional quality of forage crops. Front. Plant Sci. **9**(535): 1-18.

Carrasco, M., Rozas, J.M., Barahona, S., Alcaíno, J., Cifuentes, V., and Baeza, M. 2012. Diversity and extracellular enzymatic activities of yeasts isolated from King George Island, the sub-Antarctic region. BMC Microbiology. **12**: 251.

Cassida, K.A., Griffin, T.S., Rodriguez, J., Patching, S.C., Hesterman, O.B., and Rust, S.R. 2000. Protein degradability and forage quality in maturing alfalfa, red clover, and birdsfoot trefoil. Crop Sci. **40**: 209-215.

Castell-Miller, C.V., Zeyen, R.J., and Samac, D.A. 2007. Infection and development of *Phoma medicaginis* on moderately resistant and susceptible alfalfa genotypes. Can. J. Plant Pathol. **29**: 290-298.

Chapados, J. 2020. Personal communication.

Charmley, E. 2001. Towards improved silage quality-a review. Can. J. Anim. Sci. **81**: 157-168.

Churko, J.M., Mantalas, G.L., Snyder, M.P., and Wu, J.C. 2013. Overview of high throughput sequencing technologies to elucidate molecular pathways in cardiovascular diseases. Circ. Res. **112**(12).

Clark, J. 2003. Making quality silage bales. Focus on Forage. **6**(4).

Cline, J.C., Nelson, J.D., Gerzon, K., Williams, R.H., and Delong, D.C. 1969. In vitro antiviral activity of mycophenolic acid and its reversal by guanine compounds. Appl. Microbiol. **18**: 14-20.

Coblentz, W., and Atkins, M. 2019. Precutting bales didn't impact fermentation. Hay & Forage Grower. **34**(1): 18-19.

Coblentz, W., and Hoffman, P. 2008. Heat damaged forages: effects on forage quality. Focus on Forage. **10**(8).

Coblentz, W., Hoffman, P., and Undersander, D. 2011. Heat damaged forages: effects on forage energy content. Focus on Forage. **13**(1).

Compton, K., Molloy, W., and Jewell, L.E. 2019. Forage diseases and silage spoilage on the Avalon: a poorly characterized problem. Can. J. Plant Pathol. **41**(3): 504-506.

Copani, G., Bryan, K.A., Nielsen, N.G., Witt, K.L., Queiroz, O., Ghilardelli, F., Masoero, F., and Gallo, A. 2018. The effect of bacterial inoculant and packing density on corn silage quality and safety. XVIII International Silage Conference.

Council for Agricultural Science and Technology (CAST). 2003. Mycotoxins: Risks in Plant, Animal, and Human Systems. Task Force Report No. 139. Ames, Iowa, USA.

Crook, T.S., Stewart, B., Sims, M.B., Weiss, C.P., Coffey, K.P., Beck, P.A. 2016. The effects of moisture at baling and wrapping delay on storage characteristics of annual ryegrass round bale silage. J. Anim. Sci. **95**(1): 13.

Diaz-Nazario, D.E. 2002. Mycotoxins in feed and forages and their significance to dairy cattle production. Ph.D. thesis, North Carolina State University, Raleigh, North Carolina.

Danner, H., Holzer, M., Mayrhuber, E., and Braun, R. 2003. Acetic acid increases stability of silage under aerobic conditions. Appl. Environ. Microbiol. **69**(1): 562-567.

Dewhurst, R.J., Fisher, W.J., Tweed, J.K.S., Wilkins, R.J. 2003. Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. J. Dairy Sci. **86**(8): 2598-2611.

Duniere, L., Xu, S., Long, J., Elekwachi, C., Wang, Y., Turkington, K., Forster, R., and McAllister, T.A. 2017. Bacterial and fungal core microbiomes associated with small grain silages during ensiling and aerobic spoilage. BMC Microbiology. **17**:50.

Driehuis, F. 2013. Silage and the safety and quality of dairy foods: a review. Agr. Food Sci. **22**(2013): 16-34.

Driehuis, F., Spanjer, M.C., Scholten, J.M., and te Giffel, M.C. 2008a. Occurrence of mycotoxins in feedstuffs of dairy cows and estimation of total dietary intakes. J. Dairy Sci. **91**(11): 4261-4271.

Driehuis, F., Spanjer, M.C., Scholten, J.M., and Te Giffel, M.C. 2008b. Occurrence of mycotoxins in maize, grass and wheat silage for dairy cattle in the Netherlands. Food Addit. Contam.: Part B. **1**(1): 41-50.

Drouin, P., Tremblay, J., and Chaucheyras-Durand, F. 2019. Dynamic succession of microbiota during ensiling of whole plant corn following inoculation with *Lactobacillus buchneri* and *Lactobacillus hilgardii* alone or in combination. Microorganisms. **7**(12): 595.

Druvefors, U., Jonsson, N., Boysen, M., and Schnürer, J. 2002. Efficacy of the biocontrol yeast *Pichia anomala* during long-term storage of moist feed grain under different oxygen and carbon dioxide regimens. FEMS Yeast Res. **2**: 389-394

El-Nezami, H.S., Chrevatidis, A., Auriola, S., Salminen, S., and Mykkänen, H. 2002. Removal of common Fusarium toxins in vitro by strains of *Lactobacilus* and *Propionibacterium*. Food Addit. Contam. **19**(7): 680-686.

Eikmeyer, G., Köfinger, P., Poschenel, A., Jünemann, S., Zakrzewski, M., Heini, S., Mayrhuber, E., Grabherr, R., Pühler, A., Schwab, H., Schlüter, A. 2013. Metagenome analyses reveal the influence of the inoculant *Lactobacillus buchneri* CD034 on the microbial community involved in grass ensiling. J. Biotechnol. **167**(3): 334-343.

European Commission. 2003. Opinion of the scientific committee on animal nutrition on undesirable substances in feed. [Online]. Available: https://ec.europa.eu/food/sites/food/files/safety/docs/animal-feed_additives_rules_scan-old_report_out127.pdf [21 Apr. 2020].

European Food Safety Authority (EFSA). 2012. Scientific opinion on the risks for public and animal health related to the presence of citrinin in food and feed. EFSA J. **10**: 1-82.

Fell, J.W., Statzell, A.C., Hunter, I.L., and Phaff, H.J. 1969. *Leucosporidium* gen. n., the heterbasidiomycetous stage of several yeasts of the genus *Canidia*. Antonie van Leeuwenhoek. **35**(1969): 462-433.

Ferreras, M.C., Benavides, J., García-Pariente, C., Delgado, L., Fuertes, M., Muñoz, M., García-Marín, J.F., and Pérez, V. 2013. Acute and chronic disease association with naturally occurring T-2 mycotoxicosis in sheep. J. Comp. Path. **148**: 236-242.

Filya, I. Ashbell, G. Hen, Y., and Weinberg, Z.G. 2000. The effect of bacterial inoculants on the fermentation and aerobic stability of whole crop wheat silage. Anim. Feed Sci. Technol. **88**(1-2): 39-46.

Filya, I., Muck, R.E., and Contreras-Govea, F.E., 2007. Inoculant effects on alfalfa silage: fermentation products and nutritive value. J. Dairy Sci. **90**: 5108-5114.

Fink-Gremmels, J. 2004. Moulds and mycotoxins in silage. Pages 269-272 *in* D., Barug, H., van Egmond, R., López-García, T., van Osenbruggen, and A.,Visconti, eds. Meeting the Mycotoxin Menace. Wageningen Academic: Wageningen, The Netherlands.

Fink-Gremmels, J. 2008. The role of mycotoxins in the health and performance of dairy cows. Vet. J. **176**(1): 84-92.

Fleet, G. 1992. Spoilage yeasts. Crit. Rev. Biotechnol. **12**(1-2): 1-44.

Forwood, D.L., Hooker, K., Caro, E., Huo, Y., Holman, D.B., Meale, S.J., and Chaves, A.V. 2019. Crop sorghum ensiled with unsalable vegetables increases silage microbial diversity. Front. Microbiol. **10**: 2599.

Fredlund, E., Druvefors, U., Boysen, M.E., Lingsten, K.J., and Schnürer, J. 2002. Physiological characterisitcs of the biocontrol yeast *Pichia anomala* J121. FEMS Yeast Res. **2**: 395-401.

Frisvad, J.C., and Filtenborg, O. 1989. Analysis and screening for mycotoxins and other secondary metabolites in fungal cultures by thin-layer chromatography and high-performance liquid chromatography. Arch. Environ. Contamin. Toxicol. **18**: 331-335.

Frisvad, J. C., Smedsgaard, J., Larsen, T. O., Samson, R. A. 2004. Mycotoxins, drugs and other extrolites produced by species in Penicillium subgenus Penicillium. Stud. Mycol. **49**: 201- 241.

Gallagher, D.W., and Stefvenson, K.R. 1976. Heat damage in hay-crop silage. Ontario Ministry of Agriculture and Food. Order no. 76-007.

Gallagher, D., Parker, D., Allen, D.J., Tsesmetzis, N. 2018. Dynamic bacterial and fungal microbiomes during sweet sorghum ensiling impact bioethanol production. Bioresour. Technol. **264**: 163-173.

Gallo, A., Giuberti, G., Frisvad, J.C., Bertuzzi, T., and Nielsen, K.F. 2015. Review on mycotoxin issues in ruminants: occurrence in forages, effects of mycotoxin ingestion on health status and animal performance and practical strategies to counteract their negative effects. Toxins (Basel). **7**(8): 3057-3111.

Garon, D., Richard, E., Sage, L., Bouchart, V., Pottier, D., Lebailly, P., 2006. Mycoflora and multimycotoxin detection in corn silage: experimental study. J. Agric. Food Chem. **54**: 3479–3484.

Glushakova, A.M., Kachalkin, A.V., and Chernov, I. Y. 2015. Soil yeast communities under the aggressive invasion of Sosnowsky's hogweed (Heracleum sosnowskyi). Eurasian Soil Science. **48**(2): 201-207.

Goering, H. K., and Van Soest, P.J. 1972. Relative susceptibility of forages as affected by moisture, temperature, and pH. J. Dairy Sci. **56**(1):137-143.

Gomes, N.C.M., Fagbola, O., Costa, R., Rumjanek, N.G., Buchner, A., Mendona-Hagler, L., and Smalla, K. 2003. Dynamics of fungal communities in bulk and maize rhizosphere soil in the tropics. Appl. Environ. Microbiol. **69**(7) : 3758-3766.

Gotlieb, A. 1999. Mycotoxins in silage: a silent loss in profits. [Online]. Available: https://www.uvm.edu/pss/vtcrops/articles/Mycotoxins.html [17 Dec. 2018].

Government of Canada. 2017. Section 1: Mycotoxins in livestock feed. [Online]. Available: https://www.inspection.gc.ca/animal-health/livestock-feeds/regulatory-guidance/rg-8/eng/1347383943203/1347384015909?chap=1 [23 Apr. 2020].

Greenlees, W.J., Hanna, H.M., Shinners, K.J., Marley, S.J., and Bailey, T.B. 2000. A comparison of four mower-conditioners on drying rate and leaf loss in alfalfa and grass. Appl. Eng. Agric. **16**(1):15-21.

Han, K.J., Pitman, W.D., and Chapple, A. 2014. Moisture concentration variation of silages produced on commercial farms in the South-Central USA. Asian-Australas J. Anim. Sci. **27**(10): 1436-1442.

Han, K.J., Collins, M., Vanzant, E.S., Dougherty, C.T. 2004. Bale density and moisture effects on alfalfa round bale silage. Crop Sci. **44**(3): 914-919.

Handelsman J., Rondon M.R., Brady S.F., Clardy J., Goodman R.M. 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chem. Biol. **5**(10):R245– 249.

He, P., Young, L.G., and Forsberg, C. 1992. Microbial transformation of deoxynivalenol (vomitoxin). Appl. Environ. Microbiol. **58**: 3857-3863.

Holmes, B.J. 2000. Managing forage in tower silos. [Online]. Available: https://fyi.extension.wisc.edu/forage/files/2014/01/ManagingTowerSilos.pdf [1 Apr. 2020].

Huhnke, R.L., Muck, R.E., Payton, M.E. 1997. Round bale silage storage losses in ryegrass and legume-grass forages. Appl. Eng. Agric. **13**(4): 451-457.

Inglis, G.D., Yanke, L.J., Kawchuk, L.M., and McAllister, T.A. 1999. The influence of bacterial inoculants on the microbial ecology of aerobic spoilage of barley silage. Can. J. Microbiol. **45**(1): 77-87.

Isono, N., Haykawa, H., Usami, A., Mishima, T., and Hisamatsu, M. 2012. A comparative study of ethanol production by *Issatchenkia orientalis* strains under stress conditions. J. Biosci. Bioeng. **113**(1): 76-78.

Jančič, S., Nguyen, H.D.T., Frisvad, J.C., Zalar, P., Schroers, H.-J., Seifert, K.A., Gunde-Cimerman, N. 2015. A taxonomic revision of *Wallemia sebi* species complex. PLoS One. **10**(5).

Jani, S., Frisvad, J.C., Kocev, D., Gostinar, C., Džeroski, S., and Gunde-Cimerman, N. 2016. Production of secondary metabolites in extreme environments: food- and airborne Wallemia spp. Produce toxic metabolites at hypersaline conditions. PLoS One. **11**(12).

Jensen, T., De Boevre, M., De Saeger, S., Preußke, N., Sönnichsen, F.D., Kramer, E., Klink, H., Verreet, J.-A., and Birr, T. 2020. Effect of ensiling duration on the fate of deoxynivalenol, zearalenone and their derivatives in maize silage. Mycotoxin Res. **36**: 127-136.

Jewell, L. 2018. Personal communication.

Jiang, D., Zheng, M.L., Niu, D.Z., Zuo, S.S., Tian, P.J., Li, R.R., Xu, C.C. 2020. Effects of steam explosion pretreatment and *Lactobacillus buchneri* inoculation on fungal community of unensiled and ensiled total mixed ration containing wheat straw during air exposure. J. Appl. Microbiol. **128**(3): 675-687.

Johnston, P.R., Park, D., and Smissen, R.D. 2017. Comparing diversity of fungi from living leaves using culturing and high-throughput environmental sequencing. Mycologia. **109**(4): 643-654.

Jones, F.T., Genter, M.B., Hagler, W.M., Hansen, J.A., Mowrey, B.A., Poore, M.H., Whitlow, L.W. 1994. Understanding and coping with effects of mycotoxins in livestock feed and forage. [Online]. Available: https://projects.ncsu.edu/cals/an_sci/extension/animal/nutr/Understanding_mycotoxins.pdf [16 Apr. 2020].

Jonsson, A., and G. Pahlow. 1984. Systematic classification and biochemical characterization of yeasts growing in grass silage inoculated with Lactobacillus cultures. Anim. Res. Develop. **20**: 7-22.

Joint FAO/WHO Committee on Food Additives (JEFCA). 2001. Safety evaluations of certain mycotoxins in food. JEFCA Food Additives Series 47.

Kabisch, J., Erl-Höning, C., Wenning, M., Böhnlein, C., Gareis, M., and Pichner, R. 2016. Spoilage of vacuum-packed beef by the yeast Kazachstania psychrophila. Food Microbiol. **53**: 15-23.

Kallela, K., and Ettala, E. 1984. The oestrogenic *Fusarium* toxin (zearalenone) in hay as a cause of early abortions in the cow. Nord. Vet. Med. **36**: 305-309.

Kalač, P. 2011. The effects of silage feeding on some sensory and health attributes of cow's milk: A review. Food Chem. **125**(2): 307-317.

Karszes, J., Knoblauch, W.A., and Dymond, C. 2014. Dairy farm business summary. [Online]. Available: http://publications.dyson.cornell.edu/outreach/extensionpdf/2014/Cornell- Dyson-eb1405.pdf [1 Oct. 2018].

Katoh, K., Misawa, K., Kuma, K., Miyata, T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. **15**(30): 3059-3066.

Kelman, M. 2016. Investigating mycotoxins and secondary metabolites in Canadian agricultural commodities using high-resolution mass spectrometry. M.Sc. Thesis, University of Western Ontario, London, Ontario.

Kellerman, T.S., Marasas, W.F.O., Theil, P.G., Gelderblom, W.C.A., Cawood, M., and Coetzer, J.A.W., 1990. Leukoencephalomalacia in two horses induced by oral dosing of fumonisin B1. Onderstepoort J. Vet. Res. **57**: 269-275.

Kiessling, K.H., Pettersson, H., Sandholm, K., and Olsen, M. 1984. Metabolism of aflatoxin, ochratoxin, zearalenone, and three trichothecenes by intact rumen fluid rumen protozoa, and rumen bacteria. Appl. Environ. Microbiol. **47**: 1070-1073.

Kircher, M., and Kelso, J. 2010. High-throughput DNA sequencing – concepts and limitations. Bioessays. **32**(6): 524-536.

Klaubauf, S., Inselsbacher, E., Zechmeister-Boltenstern, S., Wanek, W., Gottsberger, R., Strauss, J., and Gorfer, M. 2010. Molecular diversity of fungal communities in agricultural soils from Lower Austria. Fungal Divers. **44**(1): 65-75.

Knutsen, A.P., Bush, R.K., Demain, J.G., Denning, D.W., Dixit, A., Fairs, A., Greenberger, P.A., Kariuki, B., Kita, H., Kurup, V.P., Moss, R.B., Niven, R.M., Pashley, C.H., Slavin, R.G., Vijay, H.M., Wardlaw, A.J., 2012. Fungi and allergic lower respiratory tract diseases. J. Allergy Clin. Immunol. **129**: 280-291.

Knutsen, H.K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Grasl-Kraupp, B., Hogstrand, C., et al. 2018. Risk to human and animal health related to the presence of 4,15-diacetoxyscirpenol in food and feed. EFSA J. **16**(8): 5367.

Koc, F., Coskuntuna, L., Ozduven, M.L., Coskuntuna, A., and Samli, H.E. 2009. The effects of temperature on the silage microbiology and aeorobic stability of corn and vetch-grain silages. Acta Agriculturae Scandinavica, Section A – Animal Science. **59**(4): 239-246.

Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A.F.S., Bahram, M., Bates, S.T., Bruns, T.D., Bengtsson-Palme, J., Callaghan, T.M., et al. 2013. Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol. **22**(21): 5271–5277.

Korn, U., Müller, M., Behrendt, U., Gossmann, M., and Ditz, M. 2005. *Fusaria* and their mycotoxins in hay and grass silage from grassland with different intensities of management. Mycotoxin Res. **21**(1): 36-39.

Kotimaa, M. 1990. Spore exposure arising from stored hay, grain and straw. Journal of Agriculture Science in Finland. **62**: 285-291.

Kunčič, M.K., Zajc, J., Drobne, D., Tkalec, Z.P., and Gunde-Cimerman, N. 2013. Morphological responses to high sugar concentrations differ from adaptations to high salt concentrations in xerophilic fungi *Wallemia* spp. Fungal Biol. **117**(7-8): 466-478.

Kung, Jr., L., and Shaver, R. 2001. Interpretation and use of silage fermentation analysis reports. Focus on Forage. **3**(13).

Kung, Jr., L. 2010. Understanding the biology of silage preservation to maximize quality and protect the environment. [Online]. Available: https://alfalfa.ucdavis.edu/+symposium/2010/files/talks/CAS05_KungBiologyOfSilage.pdf [17 October 2018].

Kung, Jr., L., Taylor, C.C., Lynch, M.P., and Neylon, J.M. 2003. The effect of treating alfalfa with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for lactating dairy cows. J. Dairy Sci. **86**(1): 336-343.

Kung, Jr. L., Shaver, R.D., Grant, R.J., and Schmidt, R.J. 2018. *Silage review*: interpretation of chemical, microbial, and organoleptic components of silages. J. Dairy Sci. **101**(5): 4020-4033.

Lacey, J. 1989. Pre- and post-harvest ecology of fungi causing spoilage of food and other stored products. J. Appl. Bacteriol. Symp. Suppl. **67**: 11S-25S.

Lafrenière, C. 2020. Personal communication.

Laganà, A. 2017. Introduction to the toxins special issues on LC-MS/MS methods for mycotoxin analysis. Toxins. **9**(10): 325.

Lamb, J.F.S., Jung, H.-J.G., Riday, H. 2012. Harvest impacts on alfalfa stem neutral detergent fiber concentration and digestibility and cell wall concentration and composition. Crop Sci. Soc. America. **52**: 2402-2412.

Lautraite, S., Bénédicte, R., Guinard, J., Parent-Massin, D. 1997. *In vitro* effects of diacetoxyscirpenol (DAS) on human and rate granulo-monocytic progenitors. Mycophathologia. **140**: 59-64.

Lavoie, K., Touchette, M., St-Gelais, D., and Labrie, S. 2012. Characterization of the fungal microflora in raw milk and specialty cheese of the province of Quebec. Dairy Sci. & Technol. **92**: 455-468.

Lea, T., Steien, K., and Størmer, F.C. 1989. Mechanism of ochratoxin A-induced immunosuppression. Mycopathologia. **107**: 153-159.

Li, Y., and Nishino, N. 2011a. Bacterial and fungal communities of wilted Italian ryegrass silage inoculated with and without *Lactobacillus rhamnosus* or *Lactobacillus buchneri*. Lett. Appl. Microbiol. **52**(4): 314-321.

Li, Y., and Nishino, N. 2011b. Effects of inoculation of *Lactobacillus rhamnosus* and *Lactobacillus buchneri* on fermentation, aerobic stability and microbial communities in whole crop corn silage. Japanese Society of Grassland Science. **57**(4): 184-191.

Lindgreen, S., Adair, K.L., and Gardner, P.P. 2016. An evaluation of the accuracy and speed of metagenome analysis tools. Scientific Reports. **6**(1).

Lindquist, J. 2017. Baleage is different than all other forage making practices. [Online]. Available:https://www.canr.msu.edu/news/baleage_is_different_than_all_other_forage_ making_practices [6 Nov. 2018].

Magan, N., and Aldred, D. 2007. Post-harvest control strategies: minimizing mycotoxins in the food chain. Food Microbiol. **119**(1-2): 131-139.

Mahanna, B., and Chase, L.E. 2003. Practical applications and solutions to silage problems. Volume 41. Pages 855-895 *in* D.R. Buxton, R.E. Muck, and J.H. Harrison, eds. Am. Soc. Agron., Crop Sci. Soc. Am., and Soil Sci. Soc. Am., Madison, WI.

Mahmoud, A.G.Y., and Zaher, E.H.F. 2015. Why nuclear ribosomal internal transcribed spacer (ITS) has been selected as the DNA barcode for fungi? Adv. Genet. Eng. **4**(2).

Malekinejad, H., Aghazadeh-Attari, J., Rezabakhsh, A., Sattari, M., and Ghasemsoltani-Momtaz, B. 2015. Neurotoxicity of mycotoxins produced in vitro by *Penicillium roqueforti* isolated from maize and grass silage. Hum. Exp. Toxicol. **34**(10): 997-1005.

Mandal, S., Van Treuren, W., White, R., Eggesbø, M., Knight, R. 2015. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microb. Ecol. Health. Dis. **26**: 27663.

Mansfield, M.A., De Wolf, E.D., and Kuldau, G.A. 2005. Relationships between weather conditions, agronomic practices, and fermentation characteristics with deoxynivalenol content in fresh and ensiled maize. Plant Dis. **89**: 1151–1157.

Mansfield, M.A., and Kuldau, G. 2007. Microbiological and molecular determination of mycobiota in fresh and ensiled maize silage. Mycologia. **99**: 269-278.

McDonald, P., A.R. Henderson and S.J.E. Heron. 1991. The biochemistry of silage. 2 ed. Chalcombe Publications, Bucks, Great Britain.

McElhinney, C., Danaher, M., Elliot, C.T., O'Kiely, P. 2015. Mycotoxins in farm silages – a 2-year Irish national survey. Grass Forage Sci. **71**: 339-352.

McEniry, J., O'Kiely, P., Forristal, P.D., and Doyle, E.M. 2006. The microbiologial and chemical composition of bales and precision-chop silages on a sample of farms in County Meath. Irish J. Agr. Food Res. **45**: 73-83.

McGinnis, M.R., and Tyring, S.K. 1996. Introduction to mycology. Medical microbiology. 4th edition. University of Texas Medical Branch at Galveston.

McNamara, K.M., O'Kiely, P., Whelan, J., Forristal, P.D., Fuller, H., and Lenehan, J.J., 2001. Vertebrate pest damage to wrapped baled silage in Ireland. Int. J. Pest. Manage. **47**(3): 167–172.

Middelhoven, W.J., and Baalen, A.H.M. 1988. Development of the yeast flora of whole-crop maize during ensiling and during subsequent aerobiosis. J. Sci. Food Agric. **42**(3): 199-207.

Middelhoven, W.J., and van Doesburg, W. 2007. Utilization of hexamethylenetetramine (urotropine) by bacteria and yeasts. Antonie van Leeuwenhoek. **91**: 191-196.

Middelhoven, W.J., de Jong, I.M., and de Winter, M. 1990. Yeasts and fungi occurring in ensiled whole-crop maize and other ensiled vegetable crops. Antonie van Leeuwenhoek. **57**: 153-158.

Mitra, S., Stärk, M., and Huson, D.H. 2011. Analysis of 16s rRNA environmental sequences using MEGAN. BMC Genomics. **12**(3): S17.

Mobashar, M., Hummel, J., Blank, R., and Südekum, K.-H. 2010. Ochratoxin A in ruminants- a review on its degradation by gut microbes and effects on animals. Toxins (Basel). **2**(4): 809-839.

Mohr, A.I., Lorenz, I., Baum, B., Hewicker-Trautwein, M., Pfaffl, M., Dzidic, A., Meyer, H.H., Bauer, J., and Meyer, K. 2007. Influence of oral application of mycophenolic acid on the clinical health status of sheep. J. Vet. Med. A. **54**(2007): 76-81.

Monnet, C., Landaud, S., Bonnarme, P., and Swennen, D. 2015. Growth and adaptation of microorganisms on the cheese surface. FEMS Microbiol. Lett. **362**(1): 1-9.

Moon, N.J., and Ely, L.O. 1979. Identification and properties of yeasts associated with the aerobic deterioration of wheat and alfalfa silages. Mycopathologia. **69**(3): 153-156.

Moore, K.J., Collins, M., Nelson, J., and Redfearn, D.D., eds. 2020. Forages, Volume 2: The science of grassland agriculture. John Wiley & Sons Ltd, Chicester, West Sussex, UK.

Moore, K.J., and Jung, H.-J.G. 2001. Lignin and fiber digestion. J. Range Manage. **54**: 420-430.

Muck. R. E. 1989. Initial bacterial numbers on lucerne prior to ensiling. Grass Forage Sci. **44**: 19-25.

Muck, R.E. 2010. Silage microbiology and its control through additives. R. Bras. Zoot. **39**: 183-191.

Muck, R.E., and Huhnke, R.L. 1995. Oxygen infiltration from horizontal silo unloading practices. Trans. ASAE. 38: 23-31.

Muck, R.E., and Shinners, K.J. 2001. Conserved forage (silage and hay): progress and priorities. Pages 753-762 *in* Proc. Int. Grassl. Congr, 19; Sao Pedro, Sao Paulo, Brazil.

Müller, H.M., and Amend, R. 1997. Formation and disappearance of mycophenolic acid, patulin, penicillic acid and pr toxin in maize silage inoculated with *Penicillium roqueforti*. Arch. Anim. Nutri. **50**: 213-225.

Murdoch, J.C., Balch, D.A., Holdsworth, M.C., and Wood, M. 1955. The effect of chopping, lacerating, and wilting of herbage on the chemical composition of silage. J. Brit. Grassland Soc. **10**: 181-188.

Mysterud, I., Høiland, K., Koller, G., and Stensrud, Ø. 2007. Molecular characterization and evaluation of plant litter-associated fungi from the spring 'grazing corridor' of a sheep herd vulnerable to alveld disease. Mycopahologia. **164**: 201-215.

Neal, J.S., Eldridge, S.M., Fulkeson, W.J., Lawrie, R., and Barchia, I.M. 2013. Differences in soil carbon sequestration and soil nitrogen among forages used by the dairy industry. Soil Biol. Biochem. **57**(2013): 542-548.

Ni, K., Minh, T.T., Tu, T.T.M., Tsuruta, T., Pang, H., and Nishino, N. 2017. Comparative microbiota assessment of wilted Italian ryegrass, whole crop corn, and wilted alfalfa silage using denaturing gradient gel electrophoresis and next-generation sequencing. Appl. Microbiol. Biotechnol. **101**: 1385-1394.

Nicholson, J.W.G., McQueen, R.E., Charmley, E., and Bush, R.S. 1991. Forage conservation in round bales or silage bags: effect on ensiling characteristics and animal performance. Can. J. Anim. Sci. **71**: 1167-1180.

Nielsen, K.F., Sumarah, M.W., Frisvad, J.C., and Miller, J.D. 2006. Production of metabolites from the *Penicillium roqueforti* complex. J. Agric. Food Chem. **54**: 3756-3763.

O'Brien, M., Nielsen, K.F., O'Kiely, P. Forristal, P.D., Fuller, H.T., and Frisvad, J.C. 2006. Mycotoxins and other secondary metabolites produced in vitro by *Penicillium paneum* Frisvad and *Penicillium roqueforti* Thom isolated from baled grass silage in Ireland. J. Agric. Food Chem. **54**: 9268-9276.

O'Brien, M., O'Kiely, P., Forristal, P.D., and Fuller, H.T. 2005. Fungi isolated from contaminated baled grass silage on farms in the Irish Midlands. FEMS Microbiol. Lett. **247**(2): 131-135.

O'Brien, M., O'Kiely, P., Forristal, P.D., and Fuller, H.T. 2007a. Quantification and identification of fungal propagules in well-managed baled grass silage and in normal on-farm produced bales. Anim. Feed Sci. Technol. **132**: 283-297.

O'Brien, M., O'Kiely, P., Forristal, P.D., and Fuller, H.T. 2007b. Visible fungal growth on baled grass silage during the winter feeding season in Ireland and silage characteristics associated with the occurrence of fungi. Anim. Feed Sci. Tech. **139**: 234-256.

O'Brien, M., O'Kiely, P., Forristal, P.D., Fuller, H.T. 2008. Fungal contamination of big-bale grass silage on Irish farms: predominant mould and yeast species and features of bales and silage. Grass and Forage Sci. **63**: 121-137.

Ogunade, I.M., Martinez-Tuppia, C., Queiroz, O.C.M. Jiang, Y., Drouin, P., Wu, F., Vyas, D., and Adesogan, A.T. 2018. *Silage Review*: Mycotoxins in silage: occurrence, effects, prevention, and mitigation. J. Dairy Sci. **101**: 4034-4059.

Ojeda, J.J., Pedro Caviglia, O., Nicolas Irisarri, J.G., and Graciela Agnusdei, M. 2018. Modelling inter-annual variation in dry matter yield and precipitation use efficiency of perennial pastures and annual forage crops sequences. Agric. For. Meteorol. **259**: 1-10.

O'Kiely, P., Forristal, D.P., Bradly, K., McNamara, K., Leneham, J.J., Fuller, H., Whelan, J. 2002. Improved technologies for baled silage. Beef Production Series No. 50. Grange Research Centre, Dunsany, Co., Meath, Ireland.

Oulas, A., Pavloudi, C., Polymenakou, P., Pavlopoulos, G.A. Papanikolaou, N., Kotoulas, G., Arvanitidis, C., and Iliopoulos, I. 2015. Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. Bioinform. Biol. Insights. **2015**: 9.

Pahlow, G. 1982. Verbesserung der aeroben Stabilitat von Silage durch Impfpraparate. Wirtschaftseigene Futter. **28**: 107–122.

Pahlow, G.,R.E. Muck, F. Driehuis, S.J.W.H. Oude Elferink, and S.F. Spoelstra. 2003. Microbiology of ensiling. Chapter 2. Pages 31–93 *in* D. R. Buxton, R. E. Muck, and J. H. Harrison, eds. Silage Science and Technology. Am. Soc. Agron, Madison, WI.

Pan D., Graneri J., and Bettucci L. 2009. Correlation of rainfall and levels of deoxynivalenol in wheat from Uruguay, 1997–2003. Food Addit. Contam. B. **2**(2):162–165.

Panasiuk, L., Jedziniaj, P., Pietruszka, K., Piatkowska, M., and Bocian, L. 2019. Frequency and levels of regulated and emerging mycotoxins in silage in Poland. Mycotoxin Res. **35**: 17-25.

Papadopoulos, Y.A., and McKersie, B.D. 1983. A comparison of protein degradation during wilting and ensiling of six forage species. Can. J. Plant Sci. **63**: 903-912.

Pestka, J.J. 2007. Deoxynivalenol: toxicity, mechanisms and animal health risks. Anim. Feed Sci. Technol. **137**: 283-298.

Petersoon, S., and Schnürer, J. 1998. *Pichia anomala* as a biocontrol agent of *Penicillium roqueforti* in high-moisture wheat, rye, barley, and oats stored under airtight conditions. Can. J. Microbiol. **44**: 471-476.

Petit, H.V., Lafreniere, C., and Veira, D.M. 1997. A comparison of methods to determine dry matter in silages. J. Dairy Sci. **80**(3): 558-562.

Plaizier, K. 2020. Personal communication.

Price, M.N., Dehal, P.S., and Arkin, A.P. 2010. FastTree 2 – Approximately maximum-liklihood trees for large alignments. PLoS ONE. **5**(3).

QIIME 2. 2019. Parkinson's mouse tutorial. [Online]. Available: https://docs.qiime2.org/2019.10/tutorials/pd-mice/ [19 Mar. 2020].

QIIME 2. 2020. "Moving Pictures" tutorial. [Online]. Available: https://docs.qiime2.org/2020.2/tutorials/moving-pictures/ [19 Mar. 2020].

Reed, K.F.M., and Moore, D.D. 2009. A preliminary survey of zearalenone and other mycotoxins in Australian silage and pasture. Anim. Prod. Sci. **49**: 696-703.

Renker, C., Blanke, V., Börstler, B., Heinrichs, J., Buscot, F. 2004. Diversity of *Cryptococcus* and *Dioszegia* yeasts (Basidiomycota) inhabiting arbuscular mycorrhizal roots or spores. FEMS Yeast Res. **4**(6): 597-603.

Richard, E., Heutte, N., Bouchart, V., and Garon, D. 2009. Evaluation of fungal contamination and mycotoxin production in maize silage. Anim. Feed Sci. Technol. **148**(2): 309-320.

Romão, D., Staley, C., Ferreira, F., Rodrigues, R., Sabino, R., Veríssimo, C., Wang, P., Sadowsky, M., and Brandão, J. 2017. Next-generation sequencing and culture-based techniques offer complementary insights into fungi and prokaryotes in beach sands. Mar. Pollut. Bull. **119**: 351-358.

Romero, J.J., Zhao, Y., Balseca-Paredes, M.A., Tiezzi, F., Gutierrez-Rodriguez, E., and Castillo, M.S. 2017. Laboratory silo type and inoculation effects on nutritional composition, fermentation, and bacterial and fungal communities of oat silage. J. Dairy Sci. **100**(3):1812-1828.

Romero, J.J., Joo, Y., Park, J., Tiezzi, F., Gutierrez-Rodriguez, E., and Castillo, M.S. 2018. Bacterial and fungal communities, fermentation, and aerobic stability of conventional hybrids and brown midrib hybrids ensiled at low moisture with or without a homo- and heterofermentative inoculant. J. Dairy Sci. **101**(4): 3057-3076.

Rojas, E.C., Sapkota, R., Jensen, B., Jørgensen, H.J.L., Henriksson, T., Jørgensen, L.N., Nicolaisen, M., and Collinge, D.B. 2020. Fusarium head blight modifies fungal endophytic communities during infection of wheat spikes. Microb. Ecol. **79**: 397-408.

Rotz, C.A., and Muck, R.E. 1994. Changes in forage quality during harvest and storage. Pages 828-868 *in* Fahey, Jr., G.C. et al. (ed.). Forage Quality Evaluation, and Utilization. Am. Soc. Agron. Madison, WI.

Rotz, C.A, and Sprott, D.J. 1984. Drying rates, losses and fuel requirements for mowing and conditioning alfalfa. Trans. ASAE **27**:715-720.

Rozpędowska, E., Galafassi, S., Johansson, L., Hagman, A., Piškur, J., and Compagno, C. 2011. *Candida albicans*– a pre-whole genome duplication yeast – is predominantly aerobic and a poor ethanol producer. FEMS Yeast Res. **11**(3): 285-291.

Ruppel, K.A., Pitt, R.E., Chase, L.E., and Galton, D.M. 1995. Bunker silo management and its relationship to forage preservation on dairy farms. J. Dairy Sci. 78: 141-153.

Russell, R. 2010. How will climate change affect mycotoxins in food? Food Res. Int. **43**(7): 1902-1914.

Sanger, F., Nicklen, S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**(12): 5463-5467.

Santos, M.C., Golt, C., Joerger, R.D., Mechor, G.D., Mourão, B., and Kung Jr., L. 2017. Identification of the major yeasts isolated from high moisture corn and corn silages in the United States using genetic and biochemical methods. J. Dairy Sci. **100**(2): 1151-1160.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., and Weber, C.F. 2009. Introducing mothur: open-source platform-independent, community-supported software for describing microbial communities. Appl. Environ. Microbiol. **75**(23): 7537-7541.

Schneweis, I., Meyer, K., Hörmansdorfer, S., and Bauer, J., 2001. Metabolites of *Monascus ruber* in silages. J. Anim. Physiol. Anim. Nutr. **85**: 38–44.

Schneweis, I., Meyer, K., Hörmansdorfer, S., and Bauer, J. 2000. Mycophenolic acid in silage. Appl. Environ. Microbiol. **66**(8): 3639-3641.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., and Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. Proc. Natl. Acad. Sci. USA. **109**: 6241-6246.

Schiro, G., Colangeli, P., and Müller, M.E.H. 2019. A metabarcoding analysis of the mycobiome of wheat ears across a topographically heterogeneous field. Front. Microbiol. **10**: 2095.

Scudamore, K.A., and Livesey, C.T. 1998. Occurrence and significance of mycotoxins in forage crops and silage: a review. J. Sci. Food Agric. **77**:1-17.

Skaar, I. 1996. Mycological survey and characterisation of the mycobiota of big bale grass silage in Norway. Ph.D. thesis, Norwegian College of Veterinary Medicine, Norway.

Skou, J.P. 1986. Notes on the habitats, morphology and taxonomy of spore cyst fungi. Apimondia **30**: 260–264.

Skladanka, J., Adam, V., Zitka, O., Mlejnkova, V., Kalhotka, L., Horky, P., Konecna, K., Hodulikova, L., Knotova, D., Balabanova, M., Slama, P., and Skarpa, P. 2017. Comparison of biogenic amines and mycotoxins in alfalfa and red clover fodder depending on additives. Int. J. Environ. Res. Public Health. **14**(4): 418.

Skladanka, J., Adam, V., Dolezal, P., Nedelnik, J., Kizek, R., Linduskova, H., Mejia, J.E.A., and Nawrath, A. 2013. How do grass species, season and ensiling influence mycotoxin content in forage? Int. J. Environ. Res. Public Health. **10**: 6084-6095.

Slatko, B.E., Gardner, A.F., and Ausubel, F.M. 2018. Overview of next generation sequencing technologies. Curr. Protoc. Mol. Biol. **122**(1): e59.

Squire, R.A., 1981. Ranking animal carcinogens: a proposed regulatory approach. Science. **214**: 877-880.

Stanciu, O., Juan, C., Berrada, H., Miere, D., Loghin, F., and Mañes, J. 2019. Study on trichothecene and zearalenone presence in Romanian wheat relative to weather conditions. Toxins. **11**(3): 163.

Statistics Canada. 2016a. Table 32-10-0166-01. Farms classified by farm type, historical data. [Online]. Available: https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210016601 [5 Nov. 2018].

Statistics Canada. 2016b. Table 32-0416-01. Hay and field crops. [Online]. Available: https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210041601&pickMembers%5B0%5D=1.1&pickMembers%5B1%5D=2.23 [5 Nov. 2018].

Statistics Canada. 2018. Table 32-10-0130-01. Number of cattle, by class and farm type. [Online]. Available: https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210013001&pickMembers%B0%5D=1.1&pickMembers%5B1%5D=2.3 [5 Nov. 2018].

Storm, I.M.L.D., Kristensen, N.B., Raun, B.M.L., Smedsgaard, J., and Thrane, U. 2010. Dynamics in the microbiology of maize silage during whole-season storage. J. Appl. Microbiol. **109**(3): 1017-1026.

Sumarah, M.W., Miller, J.D., and Blackwell, B.A. 2005. Isolation and metabolite production by *Penicillium roqueforti*, *P. paneum* and *P. crustosum* isolated in Canada. Mycopathologia. **159**: 571-577.

Tofalo, R., Fasoli, G. Schirone, M., Perpetuini, G., Pepe, A., Corsetti, A., Suzzi, G. 2014. The prominence, biodiversity and biotechnical properties of *Kluyveromyces marxianus* in the production of Pecorino di Farindola cheese. International Journal of Food Microbiology. **187**: 41-49.

Undersander, D., and Saxe, C. 2013. Field drying forge for hay and haylage. Focus on Forage. **12**(5).

Undersander, D., Wood, T., and Foster, W. 1998. Successful wrapping and storage of square bales. [Online]. Available: https://fyi.extension.wisc.edu/forage/files/2016/09/Successful-Wrapping-and-Storage-of-Square-Bales.pdf [30 Mar. 2020].

Undi, M., Wittenberg, K.M., and Holliday, N.J. 1997. Occurrence of fungal species in stored alfalfa forage as influenced by moisture content at baling and temperature during storage. Can. J. Anim. Sci. **77**(1): 95-103.

UNITE Community. 2019. UNITE QIIME release for eukaryotes 2. Version 18.11.2018. UNITE Community.

Usleber, E., Dade, M., Schneider, E., Dietrich, R., Bauer, J., and Märtlbauer, E. 2008. Enzyme immunoassay for mycophenolic acid in milk and cheese. J. Agric. Food Chem. **56**: 6857-6862.

VandeHaar, M.J., and St-Pierre, N. 2006. Major advances in nutrition: relevance to the sustainability of the dairy industry. J. Dairy Sci. **89**(4): 1280-1291.

Van Biert P. Dairy cost study: the economics of milk production in Alberta Volume 75. Economics Section, Economics and Competitiveness Branch, Alberta Agriculture and Forestry. 2017. [Online]. Available: https://open.alberta.ca/publications/1707-5084 [25 Sept. 2018].

Van Soest, P.J., Robertson, J.B., and Lewis, B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci., **74**: 3583–3597.

Vicente, F., Rodríguez, M.L., Martínez-Fernández, A., Soldado, A., Argamentería, A., Peláez, M., and de la Roza-Delgado, B. 2014. Subclinical ketosis on dairy cows in transition period in farms with contrasting butyric acid contents in silages. Sci. World J. **2014**: 279614.

Vishniac, H. 2002. *Cryptococcus tephrensis*, sp. nov., and *Cryptococcus heimaeyensis*, sp. nov.; a new anamorphic basidiomycetous yeast species from Iceland. Can. J. Microbiol. **48**: 463-467.

Voss, K.A., Plattner, R.D., Bacon, C.W., and Norred, W. 1990. Comparative studies of heptatoxicity and fumonisin B_1 and B_2 content of water and chloroform/methanol extracts of *Fusarium moniliforme* strain MRC 826 culture material. Mycopathologia. **112**: 81-92.

Vural, H.C. 2010. Genetic identification of soybean [*Glycine Max* (L.) Merr] growing in Turkey for molecular breeding using molecular markers. Biotechnol. Biotechnol. Equip. **24**(3): 2004-2008.

Wambacq, E., Latré, J.P., and Haesaert, G. 2013. The effect of *Lactobacillus buchneri* inoculation on the aerobic stability and fermentation characteristics of alfalfa-ryegrass, red clover and maize silage. Agric. Food Sci. **22**: 127-136.

Wang, H., Hao, W., Ning, T., Zheng, M., and Xu, C. 2018. Characterization of culturable yeast species associating with whole crop corn and total mixed ration silage. Asian-Australas J. Anim. Sci. **31**(2): 198-207.

Weaver, G.A., Kurtz, H.J., Behrens, J.C., Robison, T.S., Seguin, B.E., Bates, F.Y., Mirocha, C.J. 1986. Effects of zearalenone on the fertility of virgin dairy heifers. Am. J. Vet. Res. **47**: 1395-1397.

Weinberg, Z.G., and Ashbell, G. 2003. Engineering aspects of ensiling. Biochem. Eng. J. **13**(2-3): 181-188.

Weinberg, Z.G., Ashbell, G., Hen, Y., and Azrieli, A. 1993. The effect of applying lactic acid bacteria at ensiling on the aerobic stability of silages. J. Appl. Bacteriol. **75**: 512-518.

White, T. J., Bruns, T.D., Lee, S.B., and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 *in* M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds. PCR Protocols: A Guide to Methods and Applications. Academic Press: San Diego, CA.

Wilkinson, J. M., and Davies, D.R. 2013. The aerobic stability of silage: key findings and recent developments. Grass Forage Sci. **68**(1): 1-19.

Wood, G.M., Mann, P.J., Lewis, D.F., Reid, W.J., Moss, M.O. 1990. Studies on a toxic metabolite from the mould *Wallemia*. Food Addit. Contam. **7**(1): 69-77.

Woolford, M.K. 1990. The detrimental effects of air on silage. J. Appl. Bacteriol. **68**: 101-116.

Wynns, A.A., Jensen, A.B., and Eilenberg, J. 2013. *Ascosphaera callicarpa*, a new species of bee-loving fungus, with a key to the genus for Europe. PLoS One. **8**(9): 1-9.

Xin, M.-x., and Zhou, P.-j. 2007. *Mrakia psychrophila* sp. nov., a new species isolated from Antarctic soil. J. Zhejiang Univ. Sci. B. **8**(4): 260-265.

Xu, S., Yang, J., Qi, M., Smiley, B., Rutherford, W., Wang, Y., and McAllister, T.A. 2019. Impact of *Saccharomyces cerevisiae* and *Lactobacillus buchneri* on microbial communities during ensiling and aerobic spoilage or corn silage. J. Anim. Sci. **97**: 1273-1285.

Xue, Z., Wang, Y., Yang, H., Li, S., Zhang, Y. 2020. Silage fermentation and in vitro degradation characteristics of orchardgrass and alfalfa intercrop mixtures as influences by forage ratios and nitrogen fertilizing levels. Sustainability. **12**(3): 871.

Yamazaki, M., and Komagata, K. 1983. An electrophoretic comparison of enzymes of ballistosporogenous yeasts. J. Gen. Appl. Microbiol. **29**: 115-143.

Yang, R.H., Su, J.H., Shang, J.J., Wu, Y.Y., Li, Y., Bao, D.P., Yao, Y.J. 2018. Evaluation of the ribosomal DNA internal transcribed spacer (ITS), specifically ITS1 and ITS2, for the analysis of fungal diversity by deep sequencing. PLoS ONE. **13**(10).

Yiannikouris, A., and Jouany, J.-P. 2002. Mycotoxins in feeds and their fate in animals: a review. Anim. Res. **51**(2002): 81-99.

Yurkov, A.M., and Kurtzman, C.P. 2019. Three new species of Tremellomycetes isolated from maize and norther wild rice. FEMS Yeast Res. **19**(2).

Zachariasova, M., Dzuman, Z., Veprikova, Z., Hajkova, K., Jiru, M., Vaclavikova, M., Zachariasova, A., Pospichalova, M., Florian, M., Hajslova, J. 2014. Occurrence of multiple mycotoxins in European feedingstuffs, assessment of dietary intake by farm animals. Anim. Feed Sci. Technol. **193**: 124-140.

Zain, M.E. 2011. Impact of mycotoxins on humans and animals. J. Saudi Chem. Soc. **15**(2):129–144.

Zhang, T., Li, L., Wang, X., Zeng, Z., Hu, Y., and Cui, Z. 2009. Effects of *Lactobacillus buchneri* and *Lactobacillus plantarum* on fermentation, aerobic stability, bacteria diversity and ruminal degradability of alfalfa silage. World J. Microbiol. Biotechnol. **25**: 965-971.

Zheng, M.L., Niu, D.Z., Jiang, D., Zuo, S.S., Xu, C.C. 2017. Dynamics of microbial community during ensiling direct-cut alfalfa with and without LAB inoculant and sugar. J. Appl. Microbiol. **122**: 1456-1470.

APPENDIX

Table A1. Supplemental sequencing information on total number of reads and ASVs for each sample.

Sample Number	Total Number of Reads	Total Number of ASVs
1	80967	54334
2	473091	392212
3	29680	16880
4	180657	115319
5	76581	44006
6	122153	90489
7	137261	27381
8	42130	22242
9	182488	50635
10	394843	116020
11	56857	31057
12	159629	102903
13	126168	78317
14	254208	173203
15	177130	140194
16	64531	37021
17	114795	20132
18	431353	257384
19	51022	34185
20	195929	98103
21	50239	28360
22	131031	102800
23	288654	486
24	63456	18276
25	92456	57530
26	314051	228147
27	27834	15866
28	117662	22451
29	107115	51235
30	61689	14129
31	182825	92062

Sample Number	Total Number of Reads	Total Number of ASVs
32	71186	34426
33	137128	47417
34	332836	113754
35	215063	114024
36	404199	156242
37	259332	142177
38	216367	143049
39	214287	98584
40	200443	111147
41	185251	38901
42	272161	175186
43	265801	120587
44	458198	277589
45	209970	125658
46	168021	97037
47	155213	99253
48	181806	112785
49	137504	96915
50	412582	81153
51	227308	130036
52	722070	108634
53	151092	85355
54	192506	68853
55	184494	90126
56	255994	6451
57	121054	42136
58	343128	135611
59	279968	107368
60	309189	118738
61	193848	118451
62	150582	92548
63	246077	161283
64	245977	179751
65	93142	51898

Sample Number	Total Number of Reads	Total Number of ASVs
66	277133	117791
67	248732	160302
68	93860	61584
69	118844	50294
70	253584	172815
71	251123	109638
72	168184	72919
73	120399	57623
74	260956	195283
75	231740	139732
76	104750	75846
77	156636	12710
78	235092	35481
79	263882	88790
80	175470	111433
81	77772	42458
82	231214	155632
83	172043	118075
84	126988	93098
85	125419	82032
86	247867	110730
87	140400	109298
88	270182	143743
89	78764	58053
90	224957	171726
91	167094	119079
92	98111	64408
94	212114	110352

Table A2. Variables measured by each test performed on silage samples in 2018.

NIR	Wet Chemistry	Feed Analyses (As Fed and DM)	Mycotoxin Detection
Moisture (%)	DM (%)	ADF (%)	DM (%)
DM (%)	pH	Ash (%)	Mycophenolic Acid
Crude Protein (%DM)	Ammonia (%DM)	Calcium (%)	Zearalenone
ADF Protein ADICP (%DM)	Lactic Acid (%DM)	Digestible Energy (Mcal/kg)	Deoxynivalenol
ADF Protein ADICP (%DM)	Acetic Acid (%DM)	DM (%)	3-acetyl-deoxynivalenol
ADF (%DM)	n-butyric acid (%DM)	Fat (%)	15-acetyldeoxynivalenol
aNDF (%DM)	i-butyric acid (%DM)	Magnesium (%)	Aflatoxin B1
Lignin (%DM)	n-valeric (%DM)	Metabolizable Energy (Mcal/kg)	Aflatoxin B2
NDFD 30 h (%DM)	i-valeric (%DM)	Moisture (%)	Aflatoxin G1
Ash (%DM)	Propionic (%DM)	Net Energy for Gain (Mcal/kg)	Aflatoxin G2
pH	1,2-Propanediol (%DM)	Net Energy for Lactation (Mcal/kg)	Fumonisin B1
Total VFA (%DM)	1-Propanol (%DM)	Net Energy for Maintenance (Mcal/kg)	Fumonisin B2
Lactic Acid (%DM)	Ethanol (%DM)	NDF (%)	HT-2
Lactic as % of total VFA	Methanol (%DM)	NFC (%)	T-2
Acetic Acid (%DM)	1,2 butanediol (%DM)	Phosphorus (%)	Ochratoxin A
Butyric Acid (%DM)	Acetone	Potassium (%)	Diacetoxyscirpenol
TDN (%DM)		Relative Feed Value	Sterigmatocystin
Legume content		Sodium (%)	
		Starch enzymatic by UV (%)	
		TDN (%)	

NIR= DM= dry matter, ADICP= acid detergent insoluble crude protein, ADF= acid detergent fiber, NDF= neutral detergent fiber, NDFD= neutral detergent fiber digestibility, VFA= volatile fatty acids, TDN= total digestible nutrients, Mcal/kg= megacalories per kilogram, NFC= non fiber carbohydrates

Table A3. Tests performed, mycotoxins detected, and metadata used in factor analysis for silage samples collected in 2018.

Sample #	Prov	Storage Type	NIR	WC	Feed Analyses Western Canada	ZEA (μg kg^{-1})	MPA (μg kg^{-1})	Legume Content (%)	Survey Received	Inoc-ulant Used	DM (%)	Harvest Date	Date coll-ected	Cond-itioner
1	NB	Bales	Yes	Yes	Yes	ND	ND	87	Yes	Yes	41.9	12-Jun	20-Feb	Yes
2	NB	Bales	Yes	Yes	Yes	ND	ND	79	Yes	No	39.5	20-Jun	19-Feb	No
3	NB	Bales	Yes	Yes	Yes	ND	ND	41	Yes	No	42.8	15-Jun	19-Feb	Yes
4	NB	Bales	Yes	Yes	Yes	ND	ND	54	Yes	No	36.1	10-Jun	19-Feb	No
5	NB	Bales	Yes	Yes	NA	ND	ND	48	Yes	No	43.6	15-Jun	14-Mar	No
6	NB	Bales	Yes	Yes	Yes	ND	ND	13	Yes	No	54.2	21-Jun	14-Mar	Yes
7	NB	Bunker	Yes	Yes	Yes	ND	ND	69	Yes	Yes	28.8	01-Jun	15-Feb	No
8	NB	Bunker	Yes	Yes	Yes	ND	ND	62	Yes	No	26.4	05-Jun	15-Feb	Yes
9	NB	Bunker	Yes	Yes	Yes	ND	ND	100	Yes	Yes	48.4	01-Jun	15-Feb	Yes
10	NB	Bunker	Yes	Yes	Yes	ND	ND	100	Yes	Yes	34.5	15-Jun	15-Feb	Yes
11	NB	Tower	Yes	Yes	Yes	ND	ND	100	Yes	No	41.6	08-Jun	14-Mar	Yes
12	NB	Tower	Yes	Yes	Yes	ND	ND	13	Yes	No	44.0	24-Jun	18-Feb	Yes
13	NB	Tower	Yes	Yes	Yes	ND	ND	43	Yes	No	59.4	04-Jun	18-Feb	Yes
14	NB	Tower	Yes	Yes	Yes	ND	ND	46	Yes	Yes	38.7	01-Jun	19-Feb	Yes
15	NB	Tower	Yes	Yes	Yes	ND	ND	100	Yes	Yes	42.8	10-Jun	20-Feb	Yes
16	NB	Tower	Yes	Yes	Yes	ND	ND	46	Yes	Yes	40.3	10-Jun	20-Feb	No
17	NB	Tower	Yes	Yes	Yes	ND	ND	14	Yes	No	35.4	15-Jun	20-Feb	No
18	NB	Tower	Yes	Yes	Yes	ND	ND	79	Yes	U	49.3	U	02-Apr	U
19	NS	Bunker	Yes	Yes	Yes	ND	ND	44	Yes	Yes	53.9	04-Jun	22-Feb	Yes
20	NS	Bales	Yes	Yes	Yes	ND	ND	30	Yes	No	45.2	11-Jun	06-Mar	No
21	NS	Bales	Yes	Yes	Yes	ND	ND	50	No	U	35.1	U	06-Mar	U
22	NS	Bales	Yes	Yes	Yes	ND	1190	27	Yes	No	40.8	04-Jun	03-Apr	Yes
23	NS	Tower	Yes	Yes	Yes	ND	ND	71	Yes	Yes	34.9	01-Jun	10-Feb	Yes
24	NS	Bales	Yes	Yes	Yes	ND	ND	91	Yes	Yes	38.3	04-Jun	20-Mar	No
25	NS	Bales	Yes	Yes	Yes	ND	ND	38	Yes	Yes	45.3	05-Jun	20-Mar	No

Sample #	Prov	Storage Type	NIR	WC	Feed Analyses Western Canada	ZEA (μg kg^{-1})	MPA (μg kg^{-1})	Legume Content (%)	Survey Received	Inoc-ulant Used	DM (%)	Harvest Date	Date coll-ected	Cond-itioner
26	NS	Bales	Yes	Yes	Yes	ND	ND	24	Yes	No	43.8	U	29-Mar	Yes
27	NS	Bales	Yes	Yes	Yes	ND	ND	58	No	U	44.1	U	01-Apr	U
28	NS	Bunker	Yes	Yes	Yes	ND	ND	66	Yes	Yes	52.0	28-May	22-Feb	No
29	NS	Bunker	Yes	Yes	Yes	ND	ND	100	Yes	No	38.0	28-May	22-Feb	No
30	NS	Bunker	Yes	Yes	Yes	ND	ND	38	Yes	U	27.6	U	22-Feb	U
31	NS	Bunker	Yes	Yes	Yes	ND	ND	99	Yes	Yes	42.9	02-Jun	27-Feb	No
32	NS	Bunker	Yes	Yes	Yes	ND	ND	60	Yes	Yes	34.5	U	05-Mar	Yes
33	NS	Bunker	Yes	Yes	Yes	ND	ND	35	Yes	No	35.6	28-May	22-Mar	No
34	NS	Tower	Yes	Yes	Yes	ND	ND	68	Yes	Yes	44.2	25-May	22-Mar	Yes
35	NS	Tower	Yes	Yes	Yes	ND	ND	51	Yes	Yes	47.6	28-May	22-Mar	Yes
36	NS	Tower	Yes	Yes	Yes	ND	ND	35	Yes	Yes	42.7	28-May	22-Feb	No
37	NS	Tower	Yes	Yes	Yes	ND	ND	92	Yes	No	29.9	11-Jun	27-Feb	Yes
38	NS	Tower	Yes	Yes	Yes	ND	ND	17	Yes	No	44.8	22-Jun	06-Mar	Yes
39	NS	Tower	Yes	Yes	Yes	ND	ND	35	Yes	Yes	32.6	17-Jun	01-Apr	Yes
40	NS	Tower	Yes	Yes	Yes	ND	ND	81	Yes	Yes	38.3	28-May	22-Mar	No
41	NS	Tower	Yes	Yes	Yes	ND	ND	80	Yes	Yes	43.1	01-Jun	19-Mar	No
42	PE	Bales	Yes	Yes	Yes	ND	ND	36	Yes	Yes	53.6	04-Jun	28-Feb	Yes
43	PE	Bales	Yes	Yes	Yes	ND	ND	100	Yes	No	43.9	10-Jun	28-Feb	U
44	PE	Bales	Yes	Yes	Yes	ND	ND	38	Yes	No	57.0	U	01-Mar	Yes
45	PE	Bunker	Yes	Yes	Yes	ND	ND	51	No	U	54.9	U	01-Mar	U
46	PE	Bunker	Yes	Yes	Yes	ND	ND	89	No	U	34.5	U	01-Mar	U
47	PE	Tower	Yes	Yes	Yes	ND	ND	62	Yes	Yes	52.2	15-Jun	28-Feb	Yes
48	PE	Tower	Yes	Yes	Yes	ND	ND	99	Yes	Yes	39.5	U	28-Feb	Yes
49	PE	Tower	Yes	Yes	Yes	ND	ND	61	Yes	No	41.8	10-Jun	01-Mar	Yes
50	PE	Ag Bag	Yes	Yes	Yes	ND	500	77	Yes	Yes	35.1	10-Jun	01-Mar	Yes
51	NL	Bunker	NA	NA	NA	ND	ND	NA	Yes	Yes	NA	22-Jun	24-Jan	Yes

Sample #	Prov	Storage Type	NIR	WC	Feed Analyses Western Canada	ZEA (μg kg^{-1})	MPA (μg kg^{-1})	Legume Content (%)	Survey Received	Inoc-ulant Used	DM (%)	Harvest Date	Date coll-ected	Cond-itioner
52	NL	Bunker	NA	NA	NA	ND	ND	NA	No	U	NA	U	28-Jan	U
53	NL	Bales	NA	NA	NA	ND	ND	NA	No	U	NA	U	28-Jan	U
54	NL	Bales	NA	NA	NA	ND	3,480	NA	No	U	NA	U	30-Jan	U
55	NL	Bunker	NA	NA	NA	ND	ND	NA	Yes	No	NA	28-Jun	30-Jan	U
56	NL	Bunker	NA	NA	NA	ND	ND	NA	Yes	Yes	NA	20-Jun	30-Jan	Yes
57	NL	Bales	NA	NA	NA	ND	2,030	NA	Yes	No	NA	20-Jun	30-Jan	Yes
58	NL	Bunker	NA	NA	NA	ND	ND	NA	Yes	Yes	NA	U	30-Jan	Yes
59	NL	Bunker	NA	NA	NA	ND	ND	NA	Yes	Yes	NA	20-Jun	30-Jan	No
60	NL	Bales	NA	NA	NA	ND	ND	NA	Yes	No	NA	03-Jul	30-Jan	U
61	QC	Tower	NA	Yes	NA	ND	ND	NA	Yes	Yes	NA	17-Jun	U	No
62	QC	Tower	NA	Yes	NA	ND	ND	NA	Yes	Yes	NA	U	U	Yes
63	QC	Tower	NA	Yes	NA	ND	ND	NA	Yes	Yes	NA	01-Jun	U	No
64	QC	Tower	NA	Yes	NA	ND	ND	NA	Yes	Yes	NA	05-Jun	U	Yes
65	QC	Bunker	NA	Yes	NA	ND	ND	NA	No	U	NA	U	U	U
66	QC	Tower	NA	Yes	NA	ND	ND	NA	Yes	No	NA	28-May	U	Yes
67	ON	Tower	Yes	Yes	Yes	ND	ND	100	Yes	Yes	34.8	U	27-Mar	Yes
68	ON	Bales	Yes	Yes	Yes	ND	ND	54	No	U	42.4	U	25-Mar	U
69	ON	Bunker	Yes	Yes	Yes	ND	ND	100	Yes	Yes	34.7	U	27-Mar	Yes
70	ON	Bales	Yes	NA	Yes	ND	ND	13	Yes	Yes	41.1	U	26-Mar	Yes
71	ON	Bunker	Yes	Yes	Yes	ND	ND	19	Yes	U	32.7	U	28-Feb	U
72	ON	Tower	Yes	Yes	Yes	ND	ND	100	No	U	47.8	U	28-Feb	U
73	ON	Bunker	Yes	NA	Yes	ND	ND	33	No	U	36.9	U	28-Feb	U
74	ON	Bales	Yes	Yes	Yes	ND	999	82	Yes	No	41.6	U	18-Jan	Yes
75	ON	Bunker	Yes	Yes	Yes	ND	ND	66	No	U	40.2	U	21-Feb	U
76	ON	Bunker	Yes	Yes	Yes	ND	ND	28	No	U	58.2	U	U	U
77	ON	Bunker	Yes	Yes	Yes	ND	930	33	No	U	35.8	U	U	U

Sample #	Prov	Storage Type	NIR	WC	Feed Analyses Western Canada	ZEA (μg kg^{-1})	MPA (μg kg^{-1})	Legume Content (%)	Survey Received	Inoc-ulant Used	DM (%)	Harvest Date	Date coll-ected	Cond-itioner
78	ON	Bales	Yes	Yes	Yes	ND	ND	78	No	U	33.3	U	21-Feb	U
79	ON	Tower	Yes	Yes	Yes	ND	ND	92	No	U	43.9	U	U	U
80	ON	Bales	Yes	Yes	Yes	ND	ND	47	No	U	79.6	U	U	U
81	ON	Bunker	Yes	Yes	Yes	ND	ND	100	Yes	Yes	60.5	U	U	Yes
82	ON	Bales	Yes	Yes	Yes	ND	ND	100	No	U	40.1	U	U	U
83	ON	Tower	Yes	Yes	Yes	ND	ND	100	Yes	Yes	66.4	U	U	Yes
84	MB	Bunker	NA	Yes	Yes	ND	ND	NA	Yes	Yes	NA	07-Jun	20-Jun	U
85	MB	Pile	NA	Yes	Yes	ND	ND	NA	Yes	No	NA	09-Jun	14-Jun	U
86	MB	Pile	NA	Yes	Yes	ND	230	NA	Yes	Yes	NA	08-Jun	15-Jun	U
87	MB	Bunker	NA	Yes	Yes	ND	290	NA	Yes	Yes	NA	14-Jun	15-Jun	U
88	MB	Bunker	NA	Yes	Yes	ND	ND	NA	Yes	No	NA	06-Jun	26-Jun	U
89	MB	Bales	NA	Yes	Yes	ND	ND	NA	Yes	No	NA	11-Jun	19-Jun	U
90	MB	Bales	NA	NA	Yes	ND	2,940	NA	Yes	No	NA	20-Jun	19-Jun	U
91	MB	Bales	NA	Yes	Yes	ND	888	NA	No	U	NA	28-Jun	19-Jun	U
92	MB	Bales	NA	NA	Yes	350	ND	NA	Yes	No	NA	14-Jun	25-Jul	U
94	MB	Pile	NA	Yes	Yes	ND	ND	NA	Yes	Yes	NA	U	08-Aug	U

Prov= province, WC= wet chemistry, NB= New Brunswick, NS= Nova Scotia, PE= Prince Edward Island, NL= Newfoundland, QC= Quebec, ON= Ontario, MB= Manitoba, NA= not available, ZEA= zearalenone, MPA= mycophenolic acid, DM= dry matter, ND= not detected at 30 μg kg^{-1}, U= unknown, Jan= January, Feb= February, Mar= March, Apr= April, Jun= June, Jul= July, Aug= August.

Note: All 2018 silage samples were collected in 2019 except for Manitoba samples which were collected in 2018.

A4. Survey Questionnaire

General Farm Characterization

1. Is your farm **[SELECT ONE ONLY]**

☐ 1 Organic

☐ 2 Conventional

2. What is the physical land location of the farm?

_____ **[RECORD ADDRESS OF FARM]**

3. What is the physical land location of the field sampled and distance from the farm (if known)?
   a. Physical Land Location: _____**[RECORD ADDRESS OF FIELD]**
   b. Distance from Farm in km: _____

4. Milk Production

Milking System	# of Milkings per Day	# of Cows Being Milked	Milked Shipped per day (liters)	Normal Fat Test	Normal Protein Test

5. Did your farm purchase forage in the past 12 months? **[SELECT ONE ONLY]**

☐ 1 No

☐ 2 Yes: **[IF YES]**: What % of your forages is purchased:

_____%

6. How many cultivated acres were allocated to annual and perennial silage crops this year?

a. Perennials		b. Annuals	
Crop	Acres	Crop	Acres

7. How often do you rotate your perennial silage crop stands? If this differs for different crops, please provide different times for the different perennial forage crops.

_____ frequency of rotating silage crop stands **[IN YEARS]**

8. Is the forage fed to dry cows stored separately from the forage fed to the milking cows? **SELECT ONE ONLY**

☐ 1 No
☐ 2 Yes

9. Does perennial forage fed to dry cows come from different fields vs forage fed to milking cows?

☐ 1 No
☐ 2 Yes: **IF YES**, how is it managed differently?

_____
_____
_____
_____

10. a. Do you have a soil nutrient management plan? **[SELECT ONE ONLY]**

☐ 1 No
☐ 2 Yes

10b. How often do you soil test: **[SELECT ONE ONLY]**

☐ 1 every year
☐ 2 every two years
☐ 3 every three years
☐ 4 less frequently than every three years
☐ 5 Never
☐ 8 Don't know

11. Are you able to provide us a copy of the soil analysis for the field sampled? **[SELECT ONE ONLY]**

☐ 1 No
☐ 2 Yes

12. What type of storage facilities do you use on the farm? **[SELECT ALL THAT APPLY]**

☐ 1 Bunker
☐ 2 Drive over pile
☐ 3 Tower silo
☐ 4 Bales
☐ 9 Other: **SPECIFY:** _____

Field Sampled

Seeding

13. What type of perennial silage was seeded?

a. Alfalfa			b. Grass			
Variety	Seed Rate	Year Seeded	Species	Variety	Seed Rate	Year Seeded

14. What type of seeding equipment was used to seed the sampled forage?

_____

☐ 8 Don't know

15. What was the percentage of legumes, grasses and weeds in the foraged sampled**?**

[RECORD %s, TOTAL SHOULD BE 100%]

Legumes %	Grass %	Weeds%

☐ 8 Don't know

16. Are you able to provide us a copy of previous forage analysis of the field being tested? **[SELECT ONE ONLY]**

☐ 1 No

☐ 2 Yes

17. Were there any signs of the following on the sampled forage at harvest?

[SELECT ALL THAT APPLY]

☐ 1 Leaf disease

☐ 2 Insect damage

☐ 3 Winter kill

☐ 4 Frost damage

☐ 5 Drought

☐ 6 No damage

☐ 8 Don't know

☐ 9 Other: **SPECIFY**: _____

Soil and Fertilization

18. Describe the soil of the sampled field: [**SELECT ONE ONLY]**

- ☐ 1 Sandy
- ☐ 2 Loam
- ☐ 3 Clay
- ☐ 4 Silt
- ☐ 8 Don't know
- ☐ 9 Other: **SPECIFY:** _____

19. What is soil pH and when did you last apply lime to the sampled field.

_____ soil pH

_____last applied lime **RECORD DATE**

- ☐ 98 Don't know

20. What type of drainage do you have on the field sampled? **[SELECT ONE ONLY]**

- ☐ 1 Tile drainage
- ☐ 2 Drainage ditches
- ☐ 3 None
- ☐ 8 Don't know

21. Did you apply commercial fertilizer, manure or both to the sampled field? **[SELECT ONE ONLY]**

- ☐ 1 Commercial fertilizer
- ☐ 2 Manure
- ☐ 3 Both
- ☐ 4 Other (like compost) **SPECIFY:** _____
- ☐ 5 None
- ☐ 8 Don't know

22. **[ASK IF COMMERCIAL FERTILIZER IN Q.21]** Provide the following details for the commercial fertilizer on the field sampled.

☐ 1 NPK composition (ex. 40-0-0): _____

☐ 2 Rate of application: _____

☐ 8 Don't know

23. **[ASK IF MANURE IN Q.21]** If manure fertilizer, check all that apply for the field sampled

Species used: **[SELECT ALL THAT APPLY]**

☐ 1 Cattle slurry

☐ 2 Cattle solid

☐ 3 Swine slurry

☐ 4 Poultry litter

☐ 8 Don't know

☐ 9 Other: **[SPECIFY: _____]**

24. Method of application **[SELECT ALL THAT APPLY]**

☐ 1 Broadcast

☐ 2 Surface banding

☐ 3 Irrigation

☐ 4 Injection

☐ 8 Don't know

☐ 9 Other: **[SPECIFY: _____]**

25. Rate of application**:[SELECT ONE AND COMPLETE]**

☐ _____tonnes/ha

~or~

☐ _____litres /ha

~or~

☐ _____gallons per acre.

26. Are you able to provide us with a manure analysis report? **[SELECT ALL THAT APPLY]**

☐ 1 No

☐ 2 Yes

Harvest

27. What was the start date of harvest for the field sampled?

_____**RECORD DATE**

☐ 8 Don't know

28. Do you…**SELECT ONE ONLY**

☐ 1 Harvest your own silage

☐ 2 Custom harvest

☐ 3 Combination of both

☐ 8 Don't know

29. What type of mower and conditioner/crimper equipment is used?

Width of Header	Mower Type	Conditioner/Crimper Used

☐ 8 Don't know

30. What time of day did you start cutting the field sampled? **[SELECT ONE ONLY]**

☐ 1 Morning

☐ 2 Mid morning

☐ 3 Afternoon

☐ 4 Late afternoon

☐ 5 Evening

☐ 8 Don't know

31. What is your average cutting height for first cut alfalfa mix silage? **[SELECT ONE ONLY]**

- ☐ <2 inches
- ☐ 1 2-4 inches
- ☐ 2 >4 inches
- ☐ 8 Don't know

32. Was the crop wilted? **[SELECT ONE ONLY]**

- ☐ 1 No
- ☐ 2 Yes, in windrows
- ☐ 3 Yes, with tedding
- ☐ 8 Don't know

33. [**ASK IF CROP WAS TEDDED IN Q32]** How many teddings occurred on field sampled?

_____ # of teddings

- ☐ 98 Don't know

34. What were the weather conditions while crop was down? [**SELECT ALL THAT APPLY]**

- ☐ 1 Rain
- ☐ 2 Sun
- ☐ 3 Wind
- ☐ 4 Overcast
- ☐ 8 Don't know
- ☐ 9 Other: [**SPECIFY:** _____]

35. At what stage did you harvest your silage crop? **SELECT ONE ONLY**

Alfalfa, most plants were at:	Grass:
☐ 1 Early bud ☐ 2 Mid bud ☐ 3 Early flower ☐ 4 Mid flower ☐ 5 Late flower ☐ 8 Don't know	☐ 1 Pre-heading ☐ 2 Early Heading ☐ 3 Mid Heading ☐ 4 Anthesis ☐ 5 After Anthesis ☐ 8 Don't know

Silage inoculants and enzymes

36. Were inoculants and enzymes added? **[SELECT ONE ONLY]**

☐ 1 No
☐ 2 Yes

IF YES:

Location of application	Product name and manufacturer	Application Rate, if different from manufacturer's recommendation
a. Forage Harvester		
b. Storage Facility		

37. On average how many cuts do you get off the sampled field and how long do you wait between cuts?

☐ 1 Average # of cuts: _____
☐ 2 Length between cuts: _____ **[SPECIFY # OF DAYS]**
☐ 8 Don't know

If Silage Sample Was Taken from a Bunker:

38. What was your theoretical chop length? _____inches ~or~ _____cm

☐ 98 Don't know

39. How is silage moved from the field to the storage facility? **SELECT ONE ONLY**

- ☐ 1 Truck – drive beside harvester
- ☐ 2 High dump to truck
- ☐ 3 High dump to tractor wagon/trailer
- ☐ 8 Don't know
- ☐ 9 Other: **[SPECIFY:** _____

40. What are the dimensions and materials of your bunker?

- ☐ 1 Width: _____
- ☐ 2 Length: _____
- ☐ 3 Height: _____
- ☐ 4 Material: _____
- ☐ 8 Don't know

41. What method do you use when filling your bunker? **SELECT ONE ONLY**

- ☐ 1 Progressive Wedge: Feed is added at a 20-30-degree angle and height and length are increased simultaneously
- ☐ 2 Full Height: The height of the storage facility is maintained while length is increased.
- ☐ 3 Full Length: The length of the storage facility is maintained while height is increased
- ☐ 8 Don't know
- ☐ 9 Other: **SPECIFY:** _____

42. What type of equipment is used to fill and pack your bunker?

_____

_____

- ☐ 98 Don't know

43. What is the wheel set up of your filling and packing equipment? **SELECT ALL THAT APPLY**

- ☐ 1 2-wheel drive
- ☐ 2 Front wheel assist
- ☐ 3 4-wheel drive
- ☐ 4 Duals
- ☐ 8 Don't know

44. Do you add weight to your equipment?

- ☐ 1 No
- ☐ 2 Yes: **IF YES**, how much is added: _____kg ~or~ _____lbs

45. Describe the method you use to compact your silage?

_____

_____

- ☐ 98 Don't know

46. How many tonnes of silage per load _____and how many minutes between loads _____?

- ☐ 98 Don't know

47. How long does packing continue each day after the last load of forage is unloaded?

_____ hours

- ☐ 98 Don't know

48. How many tonnes of forage do you ensile per day?

_____ tonnes of forage

- ☐ 98 Don't know

49. How much time elapsed between the first load of forage and covering of the bunker? **[SELECT ONE ONLY]**

- ☐ 1 Less than 1 day
- ☐ 2 At least 1 day but less than 2 days
- ☐ 3 At least two days but less than 3 days
- ☐ 4 3 days or more
- ☐ 8 Don't know

50. Do you use plastic to cover your silage? **[SELECT ONE ONLY]**

- ☐ 1 No: **IF NO**, what are you using? _____
- ☐ 2 Yes: **IF YES**, what is the thickness of the plastic? _____mm

51. Do you use an oxygen barrier? **SELECT ONE ONLY**

- ☐ 1 No
- ☐ 2 Yes

52. **[ASK IF YES IN Q50]** How are you keeping your plastic in place? **[SELECT ALL THAT APPLY]**

- ☐ 1 Tires
- ☐ 2 Bales
- ☐ 3 Waste Feed
- ☐ 4 Soil
- ☐ 8 Don't know
- ☐ 9 Other: **SPECIFY:** _____

53. [**ASK IF YES IN Q.50]** How often is plastic checked for holes and repaired? **SELECT ONE ONLY**

- ☐ 1 Daily
- ☐ 2 Couple times a week
- ☐ 3 Weekly
- ☐ 4 Less often than once per week
- ☐ 5 Never
- ☐ 8 Don't know

54. Was the sample collected from an opened bunker? **SELECT ONE ONLY**

☐ 1 No

☐ 2 Yes

55. What date did you cover the bunker (for first cut silage)? **SELECT ONE ONLY**

_____ **RECORD DATE**

☐ 8 Don't know

56. At what rate are you removing the face of the bunker? _____ inches per day ~or~ ____cm per day)

☐ 98 Don't know

57. How do you deface your bunker and how long does the face stay exposed?

a. How is the bunker is defaced _____

☐ 8 Don't know

b. Is the face re-covered after silage is removed?

☐ 1 No

☐ 2 Yes

☐ 8 Don't know

58. When opening your bunker did you notice any form of the following **[SELECT ALL THAT APPLY]**

☐ 1 Heating

☐ 2 Effluent (water run-off)

☐ 3 Smell of spoilage

☐ 4 Observed spoilage in pockets or the surface

☐ 8 Don't know

59. If signs of heating (steam) were noticed when opening the pile did they last longer than 1 hour? **[SELECT ONE ONLY]**

- ☐ 1 No
- ☐ 2 Yes
- ☐ 8 Don't know

If Silage Sample Was Taken from a Drive Over Pile

60. What was your theoretical chop length? _____inches ~or~ _____cm

- ☐ 98 Don't know

61. How is silage moved from the field to the drive over pile? **SELECT ONE ONLY**

- ☐ 1 Truck – drive beside harvester
- ☐ 2 High dump to truck
- ☐ 3 High dump to tractor wagon/trailer
- ☐ 8 Don't know
- ☐ 9 Other: **[SPECIFY:** _____

62. What are the dimensions of the pile?

- ☐ 1 Width: _____
- ☐ 2 Length: _____
- ☐ 3 Height: _____
- ☐ 8 Don't know

63. What is the base of the drive over pile? [**SELECT ONE ONLY]**

- ☐ 1 Concrete
- ☐ 2 Dirt
- ☐ 8 Don't know

64. What method do you use when filling your drive over pile? **SELECT ONE ONLY**

- ☐ 1 Progressive Wedge: Feed is added at a 20-30-degree angle and height and length are increased simultaneously
- ☐ 2 Full Height: The height of the storage facility is maintained while length is increased.
- ☐ 3 Full Length: The length of the storage facility is maintained while height is increased
- ☐ 8 Don't know
- ☐ 9 Other: **SPECIFY:** _____

65. What type of equipment is used to fill and pack your drive over pile?

_____

- ☐ 98 Don't know

66. What is the wheel set up of your filling and packing equipment? **SELECT ALL THAT APPLY**

- ☐ 1 2-wheel drive
- ☐ 2 Front wheel assist
- ☐ 3 4-wheel drive
- ☐ 4 Duals
- ☐ 8 Don't know

67. Do you add weight to your equipment?

- ☐ 1 No
- ☐ 2 Yes: **IF YES**, how much is added: _____kg ~or~ _____lbs
- ☐ 8 Don't know

68. Describe the method you use to compact your silage.

_____

_____

_____

☐ 98 Don't know

69. How much time per tonne of silage is spent compacting the silage on the drive over pile?

How many tonnes _____ with each load of feed _____and how many minutes between loads _____?

☐ 98 Don't know

70. How long does packing continue each day after the last load of feeds is unloaded?

_____ hours

☐ 98 Don't know

71. How many tonnes of forage do you ensile per day?

_____ tonnes of forage

☐ 98 Don't know

72. How much time elapsed between the first load of forage and covering of the bunker? **[SELECT ONE ONLY]**

☐ 1 Less than 1 day
☐ 2 At least 1 day but less than 2 days
☐ 3 At least two days but less than 3 days
☐ 4 3 days or more
☐ 8 Don't know

73. Do you use plastic to cover your silage? **[SELECT ONE ONLY]**

☐ 1 No: **IF NO**, what are you using? _____

☐ 2 Yes: **IF YES**, what is the thickness of the plastic? _____mm

74. Do you use an oxygen barrier? **[SELECT ONE ONLY]**

☐ 1 No

☐ 2 Yes

75. **[ASK IF YES IN Q73]** How are you keeping your plastic in place? **[SELECT ALL THAT APPLY]**

☐ 1 Tires

☐ 2 Bales

☐ 3 Waste Feed

☐ 4 Soil

☐ 8 Don't know

☐ 9 Other: **SPECIFY:** _____

76. **[ASK IF YES IN Q73]** How often is plastic checked for holes and repaired? **SELECT ONE ONLY**

☐ 1 Daily

☐ 2 Couple times a week

☐ 3 Weekly

☐ 4 Less often than once per week

☐ 5 Never

☐ 8 Don't know

77. Was the sample collected from an open drive over pile? **SELECT ONE ONLY**

☐ 1 No

☐ 2 Yes

78. What date did you cover the drive over pile for first cut silage?

_____ **RECORD DATE**

☐ 8 Don't know

79. At what rate are you removing the face of the drive over pile? _____ inches per day ~or~ _____ cm per day

☐ 98 Don't know

80. How do you deface your drive over pile and how long does the face stay exposed?

a. _____How is drive over pile defaced

☐ 98 Don't know

b. Is the face re-covered after silage is removed?

☐ 1 No

☐ 2 Yes

☐ 8 Don't know

81. When opening your drive over pile did you notice any form of the following **[SELECT ALL THAT APPLY]**

☐ 1 Heating

☐ 2 Effluent (water run-off)

☐ 3 Smell of spoilage

☐ 4 Observed spoilage in pockets or the surface

☐ 5 None

☐ 8 Don't know

82. If signs of heating (steam) were noticed when opening the pile did they last longer than 1 hour?

☐ 1 No

☐ 2 Yes

☐ 8 Don't know

If Sample Was Taken from a Tower Silo

83. What is the dimension of your silo?

a. Diameter: _____feet ~or~ _____meters

b. Height: _____feet ~or~ _____meters

☐ 8 Don't know

84. What is the material of your silo? **[SELECT ONE ONLY]**

☐ 1 Concrete

☐ 2 Steel

85. What type of equipment do you use to fill your silo?

_____

☐ 8 Don't know

86. How many tonnes on average do you add per day?

_____tonnes

☐ 98 Don't know

87. Do you close your silo at night in the middle of harvest? **[SELECT ONE ONLY]**

☐ 1 No

☐ 2 Yes

☐ 8 Don't know

88. How much time elapsed between the first load of first cut forage and closing the silo after harvest? **[SELECT ONE ONLY]**

☐ 1 Less than 1 day

☐ 2 At least 1 day but less than 2 days

☐ 3 At least two days but less than 3 days

☐ 4 3 days or more

☐ 8 Don't know

89. What date did you close the silo after first cut was done?

_____**RECORD DATE**

☐ 98 Don't know

90. At what rate, on average, do you feed out your silage? (_____kg/day)

☐ 98 Don't know

91. When you opened your tower did you notice any form of the following?

SELECT ALL THAT APPLY

☐ 1 Heating
☐ 2 Effluent (water run-off)
☐ 3 Smell of spoilage
☐ 4 Observed spoilage in pockets or the surface
☐ 5 None
☐ 8 Don't know

92. If signs of heating (steam) were noticed when opening the tower did they last longer than 1 hour? **[SELECT ONE ONLY]**

☐ 1 No
☐ 2 Yes
☐ 8 Don't know

If Sample Was Taken from a Silage Bale

93. What are the dimension and weight of each bale?

1 Height: _____feet ~or~ _____meters_
2 Width: _____feet ~or~ _____meters_
3 Weight: _____lbs ~or~ _____kg_

☐ 98 Don't know

94. What type of equipment is used for…

1 Baling: _____

2 Wrapping: _____

☐ 8 Don't know

95. Are there knives in the round baler? **[SELECT ONE ONLY]**

☐ 1 No

☐ 2 Yes

☐ 8 Don't know

96. How long, on average between baling and wrapping? **[SELECT ONE ONLY]**

☐ 1 Less than 8 hours

☐ 2 At least 8 hours and less than 12 hours

☐ 3 At least 12 hours and less than 24 hours

☐ 4 24 hours or more

☐ 8 Don't know

97. How many bales, on average, do you wrap per day?

_____ # of bales

☐ 98 Don't know

98. Are bales wrapped... **[SELECT ONE ONLY]**

☐ 1 Individually

☐ 2 Tube

☐ 3 Stacked

☐ 8 Don't know

99. Are bales kept: [**SELECT ONE ONLY]**

☐ 1 In the open: on field

☐ 2 In the open: on concrete

☐ 3 Under roof: on field

☐ 4 Under roof: on concrete

☐ 8 Don't know

100. What is the thickness of the plastic used to wrap the bales? (mm)

_____mm

☐ 98 Don't know

101. How many layers of plastic are used per bale?

_____

☐ 98 Don't know

102. How often is plastic checked for holes and repaired? [**SELECT ONE ONLY]**

☐ 1 Daily
☐ 2 Couple times a week
☐ 3 Weekly
☐ 4 Less often than once per week
☐ 5 Never
☐ 8 Don't know

103. How long did you wait before feeding out your silage bales?

_____ # of days

☐ 98 Don't know

104. At what rate, on average, do you feed out your bales (bales per day)?

_____ bales/day

☐ 98 Don't know

105. When you opened your bales did you notice any form of the following? **SELECT ALL THAT APPLY**

☐ 1 Effluent (water run-off)
☐ 2 Smell of spoilage
☐ 3 Observed spoilage in pockets or the surface
☐ 4 None
☐ 8 Don't know

106. If signs of heating (steam) were noticed when opening your bales did they last longer than 1 hour?

- ☐ 1 No
- ☐ 2 Yes
- ☐ 8 Don’t know