Filamentous fungi in wrapped forages

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Cover: Wrapped forages, opened bale of wrapped forage, green filamentous fungi, white filamentous fungi
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Abstract

Wrapped forages of higher dry matter (DM) concentrations (> 50 %), also referred to as haylage are common in Sweden and Norway. Such forages are preserved by a combination of semi-drying and anaerobic storage leading to an environment that may affect the composition of filamentous fungi differently than in hay or silage. The aim of this research was to identify fungal species in wrapped forages with higher DM concentration in relation to forage production and management factors.

In the first study, the effect of plant maturity at harvest on microbial composition of forage was investigated. The microbial composition of fresh herbage and conserved haylage was compared for three different harvest times (June, July and August) of the first cut of the season. The fungal load increased with later harvest dates in haylage, but fungal species detected in the herbage were not detected in the haylage. In the second study, bales from 124 farms were sampled, and data on production factors, chemical composition and mycotoxin presence included. Samples for analysis of fungi were taken from patches with visible fungal growth on the bale surfaces, and from drilled samples from the forage. Results showed a higher risk of fungal presence with increasing DM concentration, or if less than eight layers of polyethylene stretch film were used for wrapping. Presence of mycotoxins and their respective fungal species were not correlated ($P>0.05$). However, higher fungal counts were positively correlated with presence of mycotoxins.

Ocular inspection and cultivation for identification of fungal species is time- and labour consuming and has inherent difficulties. Therefore, identification of fungal species by extracting fungal DNA directly from forage samples is of interest. A study on three new primers in the fungal ITS (internal transcribed spacer) region for 454-sequencing was performed. Results showed that not all fungal species can be identified in the ITS-region and therefore other DNA regions are of interest.

Keywords: Filamentous fungi, mould, wrapped forages, haylage and mycotoxins.

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Filamentösa svampar i inplastat grovfoder

Sammanfattning

Inplastat vallfoder med hög torrsubstanshalt (ts-halt) (> 50 %), även kallat hösilage, är vanligt förekommande i Norge och Sverige. Hösilage konserveras genom en kombination av torkning och lufttät lagring, vilket innebär att fodret kan vara känsligt för mögeltillväxt om det inte hanteras korrekt. Syftet med denna avhandling var därför att karaktärisera mögel i inplastat vallfoder med hög ts-halt i förhållande till olika faktorer i foderproduktion och -hantering.

I den första studien undersöktes effekten av tidpunkt för skörd på mikrobiell sammansättning i fodret. Den mikrobiologiska sammansättningen i grönmassan och i hösilaget jämfördes vid tre skördetidpunkter (juni, juli och augusti), alla i första skörd. Mängden mögel ökade med senare skördetidpunkt, men de mögelarter som påvisades i grönmassan var inte desamma som påvisades i hösilage.

I den andra studien provtogs balar från 124 gårdar med tre olika metoder. Proverna analyserades för kemisk sammansättning samt för förekomst av mögel och mykotoxiner. Dessutom samlades data in om produktionsfaktorer. Resultatet visade att risken för svampförekomst var högre vid ökande ts-halt i fodret, eller om mindre än åtta lager plastfilm hade använts vid inplastning. Inga korrelationer mellan förekomst av mykotoxiner och de svampar som kan bilda mykotoxinerna kunde påvisas ($P>0.05$). Däremot fanns en positiv korrelation mellan mängden mögel och mykotoxinförekomst.

Okulär undersökning och odling för identifiering av svamparter är metoder med inbyggda svårigheter. Därför är identifiering av svamparter genom extrahering av mögel-DNA direkt från fodret av intresse. En studie utfördes där tre nya primers i svampens ITS (internal transcribed spacer) region användes i 454-sekvensering. Alla svamparter kunde inte karaktäriseras i ITS-regionen och därför är andra regioner av intresse för framtida utveckling av DNA-baserad analysmetodik.

Nyckelord: Filamentösa svampar, mögel, inplastat grovfoder, hösilage och mykotoxiner.

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Will I be here endlessly
Without a firm sense of identity?
De/Vision

Contents

List of publications

This thesis is based on the work contained in the following papers, and are referred to in the text by Roman numerals. Articles II, III and IV were performed within a collaborative project between the Department of Animal Nutrition and Management and the Department of Forest Mycology and Plant Pathology at SLU in Uppsala.

I Schenck J. and *Müller C.E. 2014. Microbial composition pre- and post-conservation of grass-dominated haylage harvested early, middle and late in the season. *Journal of Equine Veterinary Sciences* 34, 593-601.

II Schenck J., Djurle A., Jensen Funck D., Müller C., O'Brien M. and *Spörndly R. 2019. Filamentous fungi in wrapped forages determined with different sampling and culturing methods. *Grass and Forage Science* 74, 29-41.

III Schenck J, Müller C., Djurle A., Funck Jensen D., O'Brien M., Johansen A., Rasmussen P H. and *Spörndly R. 2019. Occurrence of filamentous fungi and mycotoxins in wrapped forages in Sweden and Norway and their relation to chemical composition and management (accepted in *Grass and Forage Science*).

IV Ihrmark K., Bödeker I.T., Cruz-Martinez K., Friberg H., Kubartova A., Schenck J., Strid Y., Stenlid J., Brandström-Durling M., Clemmensen K.E. and *Lindahl B.D. 2012. New primers to amplify the fungal ITS2 region – evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiol Ecol* 82, 666-677.

The contribution of Jessica Schenck to the papers included in this thesis was as follows:

I Co-author planned the study. Collected samples and carried out laboratory work. Analysed data and did statistical analysis together with co-author. Wrote manuscript together with co-author.

II Planned the study together with co-authors. Collected field samples, carried out laboratory work, analysed data and wrote manuscript together with co-authors.

III Planned the study together with co-authors. Collected field samples, carried out laboratory work, analysed data and wrote manuscript together with co-authors.

IV Contributed samples to the study which included filamentous fungi present in stored feed. Collected samples, carried out laboratory work and contributed to manuscript.

Abbreviations

3-ACDON	3-acetyldeoxynivalenol
Ammonia-N	Ammonia-nitrogen
a_w	Water activity
BEAU	Beauvericin
CFU	Colony forming units
DON	Deoxynivalenol
DM	Dry matter
EFSA	European Food Safety Authority
ENN-B	Enniatin B
Fungi	Filamentous fungi
GLM	General linear model
Haylage	Wrapped forage with dry matter contents >500 g per kg
HTS	High-throughput sequencing
ITS	Internal transcribed spacer
LAB	Lactic acid bacteria
Mould	Filamentous fungi
NIV	Nivalenol
PCR	Polymerase chain reaction
ZEA	Zearalenone

1 Background

1.1 Forage and forage preservation

Forages in Scandinavian countries generally consists of grasses or grass and clover mixtures. Pasture grass for grazing is not available during the winter in northern hemisphere countries, and therefore forage needs to be harvested in summer and preserved for winter-feeding to ruminants and horses.

There are different ways of preserving forage, and traditionally drying to hay has been the most common method. However, hay-making is sensitive to moist weather conditions during both harvest and storage (Hlödversson, 1985). Moist conditions can cause growth of both filamentous fungi (moulds) and yeast (unicellular fungi) (Deacon, 2005), which may result in impaired hygienic quality of the forage (Hlödversson, 1985).

Hay has been completely replaced by silage for ruminants and partly replaced by wrapped forage of higher dry matter (DM) for horses (Spörndly and Nilsdotter-Linde, 2011). High DM wrapped forage is also referred to as haylage, which has been defined as containing at least 500 g DM per kg (Müller, 2018; Gordon *et al.*, 1961).

1.1.1 Hay

In hay production a final DM content above 840 g per kg and a water activity (a_w) below 0.70 is required to restrict growth of fungi (Lacey, 1989; Gregory *et al.*, 1963). Hay is preserved by drying the material to its final DM content in the field or by combining field- and barn-drying (air forced through the material). Barn-drying of hay has been shown to result in lower counts of colony forming units (CFU) of fungi (hereafter referred to as counts) in the hay compared to field-drying (Clevström & Ljunggren, 1984).

1.1.2 Silage

In the silage making procedure, plant material containing about 200 to 500 g DM per kg is packed and stored in an air-tight environment. The aim is to achieve anaerobic conditions by air-tight storage, and to facilitate a lactic acid fermentation (McDonald *et al.*, 1991). Silage making can be performed in silos (*e.g* bunker silos) or in bales. Bales are sealed by layers of stretch film, usually of polyethylene.

After sealing, microorganisms capable of growing in anaerobic conditions will start to proliferate and lactic acid bacteria (LAB) will rapidly turn the environment acidic, causing competing organisms to stop growing (McDonald *et al.*, 1991). The LAB exists naturally on the grass crop and is a part of the epiphytic microflora. Species of LAB are either homo-fermentative, producing only lactic acid in the fermentation process (*e.g. Lactobacillus plantarum*), or hetero-fermentative, that along with lactic acid also produces other fermentation products such as acetic acid (*e.g. Lactobacillus fermentum*) (Pahljeow & Dinter, 1987). The pH will drop as the concentration of acid increases. Since LAB is the most acid tolerant microorganism among its competitors, LAB continues to grow until pH is sufficiently low to stop all microbial activity at the present water activity (McDonald *et al.*, 1991).

1.1.3 Haylage

The use of haylage has increased in Sweden, (Müller, 2018; Enhäll *et al.*, 2011), Germany (Schwartz *et al.*, 2005), Finland (Saastamoinen & Hellämäki, 2012) and Norway (Vik & Farstad, 2012) as a feed for horses. Haylage is, like baled silage, wrapped in polyethylene stretch film to create an air-tight storage. However, the conservation of haylage is based on semi-drying and anaerobic storage rather than ensiling.

Due to the higher DM content and low water activity in haylage, fermentation is restricted compared to what is observed in silage. Therefore, haylage generally contains lower concentrations of fermentation products such as lactic acid, acetic acid, butyric acid and ammonia. As lactic acid production is restricted in haylage it has a higher pH value compared to silage (Müller, 2005; Jackson, 1970; Finner, 1966). Consequently, pH cannot be used as a fermentation quality variable in haylage in the same way as for silage.

1.2 Undesirable microorganisms and their metabolites

Undesirable microorganisms are present on the crop in the field or in the soil. Such microorganisms can produce metabolites such as mycotoxins or bacterial

toxins in the field and/or during storage. Many of the metabolites are potentially toxic for farm animals, equines and humans (Scudamore & Livesey, 1998).

1.2.1 Growth of undesirable microorganisms in the field

Usually bacteria colonize the crop first, followed by yeast and filamentous fungi, which will grow during plant growth and particularly during senescence (Lacey, 1989). As the forage crop matures during the growing season, the microclimate of the sward changes, resulting in an increased general microbial load on the crop (Fehrmann & Müller, 1990).

The standing crop could be infected with different fungi especially species within the genera *Cladosporium*, *Alternaria* and *Fusarium*, also referred to as field fungi. Some of these fungi may produce mycotoxins during certain conditions, *e.g.* if the crop becomes stressed due to cold or dry weather (Scudamore & Livesey, 1998; Fehrmann & Müller, 1990). Almost all field fungi can grow between 0 to 30 °C, and some species have the ability to grow at temperatures over 35 °C (Lacey, 1989).

1.2.2 Growth of undesirable bacteria after sealing

After sealing, anaerobiosis is reached quickly in silage (Pauly, 2014), which is an important factor to avoid growth of undesirable bacteria. Another crucial factor is a low pH (McDonald *et al.*, 1991). If the pH is not lowered fast enough, undesirable bacteria capable of surviving anaerobic conditions such as species within the genera *Enterobacteriaceae*, *Clostridium* and some *Bacillus* (McDonald *et al.*, 1991) could start to grow.

Bacterial species within the genus *Clostridium* could have adverse effects on the hygienic quality of silage, for example growth of *Clostridium tyrobutyricum* can cause a second fermentation of lactic acid and/or glucose which then are converted to butyric acid (McDonald *et al.*, 1991). Another clostridial species, *Clostridium botulinum,* could have adverse effects on animal health by producing the toxin botulin causing the fatal disease botulism (Johnson *et al.*, 2010; Roberts, 1988).

1.2.3 Growth of fungi in wrapped forage

Common storage fungi present in wrapped forages are within the *Aspergillus*, *Fusarium* and *Penicillium* genera (Driehuis *et al.*, 2018; Wilkinson, 1999). Different fungal species have different optimal condition for growth. Water availability for fungi is determined by the proportion of free water in the forage. The optimal water activity differs depending on fungi genera (Nelson, 1993;

Magan & Lacey, 1988). Maximum water availability (1.00 a_w) is not always optimal for all fungi. For example, *Eurotium* species have the highest growth rate of 0.90 to 0.95 a_w and some *Penicillium* species at around 0.98 a_w (Magan & Lacey, 1988).

Yeast can grow both in aerobic and anaerobic conditions. In silage, where the conditions are anaerobic, yeast ferment sugars (glucose and fructose) to ethanol and carbon dioxide (CO_2). If oxygen is present, some yeast species use lactic acid as an energy substrate and degrade it to CO_2 and H_2O, which will result in a higher pH (McDonald *et al.*, 1991). The harvested forage can be infected or contaminated with different fungi *e.g.* species within the storage flora such as *Penicillium* spp. and *Aspergillus* spp. (Lacey, 1989). The storage fungi can grow in a wide range of temperatures from -4 °C (*e.g*. *Penicillium aurantiogriseum*) to 35 °C (*e.g. Aspergillus fumigatus*) (Lacey, 1989). Other physico-chemical variables that affect fungal growth are *e.g*. presence of oxygen, pH and substrate availability (energy sources and nitrogen) (Yiannikouris & Jouany, 2002; Nelson, 1993). Additionally, undissociated forms of acids could inhibit growth of fungi (Woolford, 1990).

For wrapped forage, growth of storage fungi is prevented primarily through exclusion of oxygen and to some extent by the low pH environment (Borreani & Tabacco, 2008; Clarke, 1988). If oxygen-rich air enters through the polyethylene stretch film surrounding the bales, growth of fungi will most likely occur.

Oxygen may enter the forage through damage in the polyethylene stretch film (O'Brien *et al.*, 2008; O'Brien *et al.*, 2007), insufficient overlapping of the film layers (Bolsen, 2006), poor quality of the polyethylene stretch film, or insufficient glue between film layers (Paillat & Gaillard, 2001). The occurrence of fungal growth in wrapped forages with higher DM contents may be higher compared to wrapped forages with low DM contents (O'Brien *et al*., 2008). In haylage the risk of punctures in the polyethylene stretch film is probably higher compared to baled forages with lower DM contents, as drier grass is harder and sharper and does not bend to pressure in the same way as grass with higher moisture content (Behrendt *et al.*, 1997).

1.3 Filamentous fungal species in forages

Presence of filamentous fungi in forages is undesired for two main reasons; the presence of spores, and the potential risk of mycotoxin production. Some fungal species produce spores which can lead to illness in animals and humans (Driehuis *et al*., 2018). One example is *A. fumigatus* that can cause aspergillosis (Tell, 2005). If mycotoxins are present in the feed it could be hazardous to the

animals themselves, but they can also be transmitted to human food of animal origin (Driehuis *et al.*, 2018).

However, not all fungi produce hazardous spores or mycotoxins, and therefore some species are more important than others in animal feed. (Driehuis *et al.*, 2018; Wilkinson, 1999). Several of potentially dangerous fungal species within the *Aspergillus*, *Fusarium* and *Penicillium* genera have previously been found in wrapped forages (Driehuis *et al.*, 2018; Wilkinson, 1999).

1.3.1 *Alternaria* species

One of the most common species in this genus is *Alternaria alternara* (Ostry, 2008). Optimal growth temperature for *A. alternara* is around 25 °C at 0.98 a_w and it has been shown that the species can grow at low oxygen levels (Häggblom, 1981; Magan *et al*., 1984; Ostry, 2008). Species within the genus *Alternaria* are common field fungi and can be found in soil but also in foodstuffs (Ostry, 2008). *Alternaria* spp. have previously been identified in alfalfa hay in Canada (Undi & Wittenberg, 1996), and *A. alternara* has been identified in wrapped forages in Norway (Skaar, 1996).

1.3.2 *Aspergillus* species

Fungi within *Aspergillus* spp. belong to the storage mycoflora and can produce spores that may cause mycoses or allergies, as well as mycotoxins (Geiser *et al.*, 2007). *Aspergillus fumigatus* is common world-wide and grows predominantly in warm climates (Pitt, 2000), but has also been found in colder climates (Samson *et al.*, 2010). In silage, *A. fumigatus* is often associated with spoilage and heating possibly initiated by other microorganisms (Scudamore & Livesey, 1998). *Aspergillus* species that have been detected in hay include *A. glaucus, A. flavus, A. fumigatus* and *A. versicolor* (Wittenberg *et al.*, 1996), and in wrapped forages *e.g. A. fumigatus, A. flavus* and *A. candidus* (Skaar, 1996).

1.3.3 *Fusarium* species

One common group of fungi that are frequently found in the field flora are the *Fusarium* species (Scudamore & Livesey, 1998). Generally, *Fusarium* species prefer to grow in moist and cool conditions (Richard, 2007). Species have been detected in maize silage, *e.g. F. verticillioides* (González Pereyra *et al.*, 2007), and in grass silage, *e.g. F. culmorum* (O'Brien *et al.*, 2008). *Fusarium* species have also been detected in wrapped forages in Sweden (Müller *et al.*, 2011) and in Norway (Skaar, 1996). One of the most prevalent species in the Nordic countries is *F. avenaceum* common in grain crops (Jestoi, 2008).

1.3.4 *Mucorales*

Fungi in the order *Mucorales* are usually fast growing with typically anamorphic sporangiospores (Samson *et al.*, 2010). Growth predominately occurs on decaying organic material (Hoffmann *et al*., 2013). Species within *Mucorales* are considered non-toxin producers (or have a weak biotoxic activity) (Reiss, 1993). Mucoraceous moulds have been found in silage in Ireland (O'Brien *et al.*, 2005a), silage in Norway (Skaar, 1996) and in wrapped forages on Swedish horse farms (Müller *et al*., 2011).

1.3.5 *Penicillium* species

Many species within the genus *Penicillium* are isolated from soil but also from food and feed. Identification of *Penicillium* species are crucial, since many are known mycotoxin producers (Samson *et al.*, 2010). One of the most common toxicogenic fungal species in silage is *P. roqueforti* found in. Norway (Skaar, 1996), Germany (Auerbach *et al.*, 1998) and Ireland (O'Brien *et al.*, 2007; O'Brien *et al.*, 2005a). This species may grow on the silage surface and interior, and can grow under acidic conditions and under low oxygen pressure (Auerbach *et al.*, 1998). It is spore producing, and the spores can survive for a long time in the environment both indoors and outdoors (Dijksterhuis, 2017). *Penicillium* spp. are considered as storage fungi.

Visible growth of *P. roqueforti* on the surface of baled silage was reported on more than 40 % of 360 examined bales from 180 farms in an Irish field study (O'Brien *et al.*, 2008). Additionally, *P. roqueforti* has been detected in grass silage (Boysen *et al.*, 2000) and wrapped forage (haylage and silage) (Müller *et al.*, 2011) in Sweden; in baled grass silage in Norway (Skaar, 1996), and in grass silage in Canada (Sumarah *et al.*, 2005). Other *Penicillium* species have also been found in baled silage, such as. *P. paneum* identified in Ireland (O'Brien *et al.*, 2005a) and *P. purpurogenum*, *P. crustosum*, *P. melanochlorum* and *P. aurantiogriseum* in Norway (Skaar, 1996).

1.3.6 Other species

A common macrofungus in baled silage in Ireland is *Schizophyllum commune*, which produces gilled bracket mushrooms that protrude the polyethylene stretch film wrapped bales (O'Brien *et al.*, 2007; Brady *et al.*, 2005). Once *S. commune* has pushed through the film layers and emerges to surface, there is an increased risk of growth of other microorganisms (Brady *et al*., 2005). Other fungal species or genera that are of interest and that could be present in silage are *e.g.*,

Byssochlamys nivea (Puel *et al.*, 2005; Skaar 1996), *Geotrichum* spp (Skaar, 1996), and in hay *e.g*. *Wallemia sebi* (Hanhela *et al.*, 1995).

1.4 Mycotoxins in forages

Mycotoxins are fungal secondary metabolites produced by some fungal species which can be lethal or cause diseases to animals and humans and should consequently be avoided in feeds. Fungi that are potential mycotoxin producers do not always produce mycotoxins, but if environmental factors such as water activity, pH, humidity and temperature are optimal for mycotoxin production it could occur (Scudamore & Livesey, 1998).

One main of the main factors is temperature, which needs to be above freezing for mycotoxin formation to take place. Furthermore, oxygen needs to be present and DM content of the feed should be above 200 g per kg (Scudamore & Livesey, 1998). Some mycotoxigenic fungi can be found on grass and clover, *e.g*. *Alternaria* and *Fusarium* species (Di Menna & Parle, 1970), meaning that mycotoxins may be formed in the field and could therefore be present also in the harvested forage. Other mycotoxigenic fungi may occur post-harvest, and produce mycotoxins in the forage during storage (Scudamore & Livesey, 1998). Studies on mycotoxin presence in grass silage are scarce, and therefore results from grain and maize silage may provide guidance of which mycotoxins that could be if interest.

1.4.1 Toxins that can be produced by *Alternaria* species

Fungi within *Alternaria* spp. may produce up to 30 different mycotoxins (EFSA, 2011a; Ostry, 2008). Examples of mycotoxins include alternariol, alteneune, tenuazonic acid and altertoxins. Alternariol is the most common mycotoxin produced by *Alternaria* which has been found in forages (Fraeyman *et al*., 2017). In grass hay, 600 µg alternariol per kg (Séguin *et al*., 2010) and on average of 89 µg per kg (Zachariasova *et al*., 2014) has been reported. In grass silage an average of 16 µg alternariol per kg has been reported (Zachariasova *et al*., 2014).

1.4.2 Toxins that can be produced by *Aspergillus* species

Species within *Aspergillus* can produce several different mycotoxins, such as aflatoxins, gliotoxin and patulin (Samson *et al.*, 2010). There are only a few reports of presence of aflatoxins in grass silage (Scudamore & Livesey, 1998). One reason is that the acidic environment in silage is unfavourable for the growth

of *Aspergillus* species. Aflatoxin-forming ability was however reported in *A. flavus* isolated from hay in Sweden (Clevström & Ljunggren, 1984).

Gliotoxin is produced by *A. fumigatus* (Klich, 2002). The toxin has been confirmed to be present in hay, silage and straw (Scudamore & Livesey, 1998). Hay and oats have been reported as favourable substrates for the synthesis of gliotoxin. Optimal temperatures for formation of gliotoxin are 30 to 36 °C, short after harvest and also during decomposition (Scudamore & Livesey, 1998).

Patulin can be produced by several fungal species of different genera, *e.g.* within *Aspergillus* and *Penicillium* (Mostrom & Jacobsen, 2011). One examples of an *Aspergillus* species with the capacity to produce patulin is *A. clavatus* (Northolt *et al.*, 1978).

1.4.3 Toxins that can be produced by *Fusarium* species

Fusarium species may produce a variety of toxins. Deoxynivalenol (DON) is a *Fusarium* derived trichothecene, primarily produced by *F. graminearum* (Richard, 2007). Climate conditions that increase the risk of DON production in cereals are low or high amount of rainfall, and warm weather. A temperature higher than 32 °C however decreases the risk of DON production in the field (Paterson & Lima, 2011). Zearalenone (ZEA) can co-exist with DON and both *F. graminearum* and *F. culmorum* can produce ZEA (Richard, 2007). Occurrence of DON and ZEA has previously been reported in maize silage in Poland (Kosicki *et al*., 2016; Panasiuk *et al.*, 2019) and Denmark (Storm *et al*., 2010). The toxins have also been found in grass silages in Poland (Panasiuk *et al.,* 2019).

Other common trichothecene toxins found in cereals and maize silage are T-2 and HT-2 toxin (Yiannikouris & Jouany, 2002). These toxins have been found in maize silages in Switzerland (Eckard *et al*., 2011) and in Poland (Panasiuk *et al.*, 2019). The most common T-2 producing fungus is *F. sporotrichioides* (Richard, 2007) and a common HT-2 producing fungal species include *F. acuminatum* and *F. poae* (Marin *et al.*, 2013). Another example is *F. langsethiae* which can produce both T-2 and HT-2 toxin, often occuring in oat, barley and wheat (Morcia *et al*., 2016).

Other toxins that derive from *Fusarium* species are acetyldeoxynivalenol (3-ACDON) which is an acetylated precursor to DON and nivalenol (NIV), which also are trichothecene toxins (Petska, 2010; Scudamore & Livesey, 1998). These toxins may be produced by fungi within the genera *Fusarium*, *Trichoderma* and *Phomopsis* (Ogunade *et al.*, 2018). Acetyldeoxynivalenol has previously been reported in maize silages in Switzerland (Eckard *et al*., 2011) and Denmark (Storm *et al*., 2010).

There are two so-called emerging toxins; enniatin B (ENN B) and beauvericin (BEAU), produced by *e.g. F. avenaceum* and *F. culmorum,* and *F. oxysporum* and *F. verticillioides,* respectively (Fraeyman *et al*., 2017; Jestoi, 2008). Enniatin B and BEAU have been found in maize silage in Denmark (Sørensen *et al*., 2008).

1.4.4 Toxins that can be produced by *Penicillium* species

A variety of mycotoxins may be produced by different *Pencillium* species. Roquefortine C is mainly produced by *P. roqueforti* and is a mycotoxin that could have severe effects on animal health. Roquefortinie C have been found in silages in Germany (Auerbach *et al.*, 1998) and Ireland (McElhinney *et al.,* 2016). *Penicillium roqueforti* may produce the toxins patulin, roquefortine C (Auerbach *et al.*, 1998) and mycophenolic acid (Puel *et al*., 2005), among others.

The toxin patulin can also be produced by *P. brevicompactum, P. carneum, P. expansum* and *P. paneum* (Frisvad *et al.*, 2004; Auerbach *et al.*, 1998). Patulin production has been induced in samples of *P. paneum* isolated from grass silage in Ireland, indicating that patulin could be a toxin present in grass silages (O'Brien *et al.*, 2006b).

Mycophenolic acid could also be produced by *P. roqueforti* (Cheli *et al.*, 2013). This toxin has been found in grass silages in Germany (Schneweis *et al.*, 2000) and in silages in Netherlands (Driehuis *et al.*, 2008).

1.4.5 Other mycotoxins

Other mycotoxins that have been found in silage and/or hay include agroclavine, andrastin A, festuclavine and marcfortine A (*Penicillium* spp. derived toxins), cyclopiazonic acid (*Aspergillus* spp. and *Penicillium* spp. derived toxin), and monacolin (*Monascus ruber* derived toxin) (Gallo *et al.,* 2015). Furthermore, *B. nivea* can produce the toxin mycophenolic acid (Puel *et al.*, 2005).

1.5 Strategies to limit fungal growth in wrapped forages

Microbial degradation of forage is associated with both economic and animal health risks, and therefore good management practices are required during production and storage (Dunière *et al*., 2013). O'Brien *et al.* (2007) found great variation in fungal occurrence between farms, indicating that management was an important factor. One very important significant management factor was visible damage of the polyethylene stretch film (O'Brien, 2007). Factors that may damage the polyethylene stretch film are farm machinery, livestock, wild-

life, rodents and birds (O'Brien *et al*., 2008; McNamara *et al*., 2001). McNamara *et al*. (2001) reported that in Ireland most of the damages to the polyethylene stretch film of wrapped bales during storage were due to birds (63 %) and cats (29 %). If bales were stored in the field, the risk of cat damage to the bales was lower compared to storage at the farm.

Furthermore, the quality of the polyethylene stretch film is important. The film should be strong enough to resist handling, storage, and wild animals (Borreani *et al*., 2018). During storage of wrapped bales it is important that the polyethylene stretch film is kept intact, to avoid oxygen-rich air leakage into the bale and this in turn will prevent fungal growth. Storing bales in several tiers (more than three) increased damage to the polyethylene stretch film compared to storage in one or two tiers (McNamara *et al.*, 2001).

Additives have been used in silage for decades and they are usually categorized from their effects: fermentation inhibitors, fermentation stimulants, aerobic deterioration inhibitors, and absorbents and nutrients. Some additives have just one effect whereas other additives have several of these combined effects. Additives in silage are homofermentative LAB, formic acid and propionic acid. The additives can also prolong the aerobic stability and decrease DM losses (Muck *et al.*, 2018). However, additives are rarely used for the preservation of forage with high DM content (Jaakkola *et al.*, 2010).

1.6 Methods to detect filamentous fungi in forages

1.6.1 Ocular inspection

Ocular inspection for the presence of fungi is often used to evaluate of fungal presence in forage, and often in combination with olfactory sensation. However, ocular inspection as fungal detection method has been shown to have poor correlation to detection by cultivation on artificial media (Raymond, 2000). A common way to grade the fungal presence in wrapped forage bales is to measure the visible fungi on the bale surface in percentage of the total bale surface area (O'Brien *et al*., 2008; Spörndly *et al*., 2017).

1.6.2 Culturing

Culturing is a common procedure for detection of fungi in feed samples. One effective qualitative method is direct plating, where a few small pieces of the forage material is placed on agar plates with selected growth media and incubated in specific temperatures. Dilution plating is a quantitative method,

where the sample is homogenized and thereafter prepared in standard ten-fold dilution series and inoculated on selected growth media (agar plates) and incubated in specific temperatures. After incubation for a certain number of days, colony forming units (CFU) on the agar plates are counted. Also, fungal colonies present in the sample itself may be used for qualitative examination by direct plating (Samson *et al.*, 2010).

Incubation temperature

It is important to select optimal incubation temperatures as different fungal species have different temperature requirements. Species within *Aspergillus* genera have best growth options between 25 to 37° C, *Mucorales* prefer 20° C, while *Fusarium* and *Penicillum* prefer temperatures around 25° C (Samson *et al.*, 2010).

Growth media

Different fungal species have different nutrient requirements for optimal growth. Consequently, it is important to use general growth media when samples of unknown fungal species composition are examined. Two commonly used growth media are Malt Extract Agar (MEA) and Dichloran 18 % Glycerol Agar (DG18) (Samson *et al.*, 2010). Xerophilic species, such as species within the genera *Penicillium* and *Aspergillus,* prefer to grow on media with low water activity containing high concentrations of soluble carbohydrates, *e.g*. DG18 (Samson *et al.*, 2010).

1.6.3 Identification of fungal colonies

Fungal colonies can be identified based on their macromorphological (size of colony, colour and medium buckling, etc.) and micromorphological features (sporangia, sporangiophores, conidiophores, conidia, hülle cells and ascospores). Morphological identification of *Fusarium* species may be challenging since the structures of macro- and microconidia is similar between different species, and not all species are cultivable in the laboratory (Samson *et al*., 2010). Also, many fungal species do not produce spores, and spores from different species could be similar, making the identification challenging or impossible (Samson *et al*., 2010).

Additional limitations are that some fungal species are fast-growing and others are slow-growing, meaning that fast-growing fungi could dominate the agar plate and thereby hide slow growers. Also, many fungi are not cultivable on artificial media (Streit & Schmitz, 2004). One example are the plant

pathogenic group rust fungi (Aime *et al*., 2017). Therefore, other methods are needed to confirm the identification or to identify the species in question (Samson *et al.*, 2010).

An alternative to identification by fungal morphology are the use of molecular methods such as DNA sequencing (Gardes & Bruns, 1993). These may also be used as an additional tool for identification of species together with morphology methods. Fungal colonies are then re-cultured on new agar plates and a small piece of the mycelia is used for DNA extraction. Depending on the species, a selected gene that is specific for that species or genus is amplified by a polymerase chain reaction (PCR). Thereafter, the PCR-product is DNA-sequenced and compared with known sequences found in public databases such as GenBank database sequences from the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/BLAST) using the BLASTN algorithm (Altschul *et al.*, 1997).

1.6.4 High throughput sequencing

One sequencing method is large-scale parallel pyrosequencing, also referred to as 454-sequencing, which can read several hundreds of thousands sequences simultaneously (Ellegren, 2008). This sequencing method is a second generation high-throughput sequencing (HTS) method (Nilsson *et al*., 2019). In this method the DNA is extracted directly from the forage sample, and thereafter PCR is performed. The 454-sequencing method is based on emulsion clonal amplification on fibre optic chips (Margulies *et al.*, 2005). Usually the internal transcribed spacer (ITS) region is sequenced by using primer pair ITS1F and ITS4 for studies of fungal communities in 454-sequencing of environmental samples such as soil communities. Many of the fungi where 454-sequencing is used are within mycorrhizal, saprophytic and plant-pathogenic fungi (O'Brien *et al.*, 2005b). The method has not previously been used for forage samples.

2 Objectives

The overall aim of this PhD project was to identify the composition of filamentous fungi in wrapped forages of high DM contents (> 50 %), and to evaluate the impact of forage production factors on the presence of fungi and mycotoxins in such forage. More specifically, the experiments were implemented to study:

1. microbial composition in wrapped forage pre- and post-preservation,

2. sampling methods for quantitative and qualitative analysis of fungal presence in wrapped forage,

3. growth of filamentous fungi in relation to management factors and chemical composition of the crop, and

4. presence of mycotoxins in wrapped forages in relation to fungal presence, management factors and chemical composition of the crop.

3 Materials and methods

All studies (Paper I to IV) were conducted at SLU in Uppsala, Sweden. The study in Paper I and IV were conducted separately within the Department of Animal Nutrition and Management and the Department of Forest Mycology and Plant Pathology respectively. The studies in Paper II, III and IV were a collaborative project between the Department of Animal Nutrition and Management and the Department of Forest Mycology and Plant Pathology at SLU in Uppsala, Sweden between 2009 and 2013.

3.1 Herbage and wrapped forages

Fresh herbage samples used for microbial analysis in Paper I were taken from the primary growth (first cut of the season) of the same grass-dominated sward at three time points for harvest; June, July and August, during 2009. Samples from preserved forage were collected from bales of wrapped forage and used for analysis of microbial composition (Paper I).

Samples of wrapped forages for Paper II and III were collected from 124 farms in Sweden and Norway during two years; 2010 and 2011 (Figure 1). The locations of the farms represents the main grassland areas in the respective countries. Bales were sampled from 49 farms in Sweden during 2010, and from 50 farms in Sweden and 25 in Norway during 2011. Sampling was performed from April to July in 2010 and from February to June in 2011.

3.2 Sampling procedure

In Paper I, II and III, wrapped forages were sampled according to a standardized protocol, and the same sampling equipment was used. The seal integrity of wrapped bales was tested by measuring the gas entry rate (Spörndly *et al.*, 2008a). The

Figure 1. Location of 124 farms surveyed in Sweden and Norway where sampling of wrapped forage was performed in 2010 and 2011 (Paper II and III).

forage samples were obtained by core sampling, a cylindrical steel core sampler (length × inner diameter, 0.65 m × 40 mm ø) connected to an electric drill.

In Paper I, the polyethylene stretch film was removed from the bale before sampling. Thereafter, six core samples spread over the bale surface were taken from each bale and samples were mixed in a clean plastic bag to produce one sample per bale. In Paper II and III, three bales from the same harvest at each farm were randomly chosen for sampling. For one of the three bales, the polyethylene stretch film was removed and patches with visible fungal patches were measured and sampled. The polyethylene stretch film remained on two of three randomly chosen bales. Thereafter, eight core samples were taken from

each bale. The core samples were mixed in a clean plastic bag to produce one sample per bale (Figure 2).

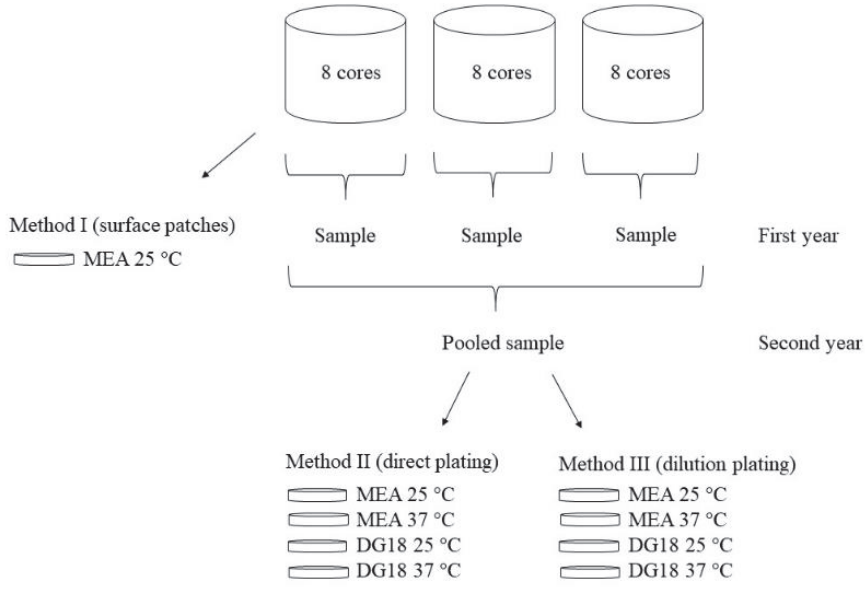

Figure 2. Sampling procedure at each farm using sampling Method I (bale surface patches), II (direct plating by core sampling) and III (dilution plating by core sampling). At each farm three bales were sampled. Eight cores per bale were taken and mixed to produce one sample per bale in 2010 (three samples per farm), and to one sample per farm in 2011 (one pooled sample from three bales per farm). Each pooled sample was used for cultivation of fungi using direct plating and dilution plating with two substrates (malt extract agar (MEA) and dichloran-glycerol agar (DG18)) and two inoculation temperatures. Visible fungi on the bale surface was also sampled and inoculated on MEA plates.

3.3 Culturing of bacteria

In Paper I, serial dilutions were used for the enumeration of LAB, clostridial spores and enterobacteria using different selective culture media. Rogosa agar (Merck, KgaA, Darmstadt, Germany) was used to culture LAB (Carlile, 1984), violet red bile dextrose agar (Merck, KgaA, Darmstadt, Germany) was used to culture enterobacteria (Seale *et al*., 1986) and reinforced clostridia medium (Merck, KgaA, Darmstadt, Germany) with the addition of cycloserine and neutral red was used for clostridial spore enumeration (Seale *et al*., 1986: Carlile, 1984).

3.4 Culturing of fungi

Samples for quantitative microbial analysis in Paper I, II and III were prepared by adding ¼ strength Ringers solution to 50 g of sample. The samples were processed for 2 x 60 seconds in a laboratory blender, and thereafter ten-fold dilution series were prepared. In Paper II and III, Tween-80 was added to the Ringer solution.

In Paper I, serial dilutions were used for enumeration of fungi. Fungi were inoculated on triplicate malt extract agar (MEA) plates (Merck, KgaA, Darmstadt, Germany) and incubated for seven days at 25 ºC. After two days, yeast colonies were counted and after five (confirmed after seven days), colonies of fungi were counted. In Paper II, the colonies were counted after five (confirmed after ten days) of incubation at 25 °C (Seale *et al*., 1986).

In Paper II and III, fungi were isolated using three methods. In Method I, direct plating of visible mycelium and/or spores from patches of mycelia on the bale surface was done using MEA plates. They were incubated for ten days at 25 °C. In Method II, direct plating of pieces from core samples of forage was performed using triplicate MEA and dichloran-glycerol agar (DG-18) plates incubated for ten days at 25 and 37 ºC. In Method III, dilution series were inoculated on triplicate MEA and DG-18 plates, which were incubated as described for Method II. Results from dilution plating was used for the calculation of numbers of colony-forming units (CFU per g) from core samples (Figure 2).

3.5 Identification of filamentous fungi

All filamentous fungi detected on original agar plates with Method I were re-inoculated on MEA plates at 25 °C and identified based on morphology and DNA sequencing.

In Method II and III, fungal colonies on original plates were selected for further analysis as following: for each farm, the counts of CFU at DG18 25 °C, DG18 at 37 °C, MEA at 25 °C and MEA at 37 °C were handled as four separate groups. Within each group, colonies sharing the same macroscopic characteristics such as colony colour, mycelium structure, medium buckling and microscopic appearance of conidia, and up to three colonies were reinoculated on new MEA plates. Thereafter the colonies were identified as follow:

The identification key described by Klich (2002) was used to identify species within the genus *Aspergillus* and the identification key by Pitt (2000) was used to identify species within the genus *Penicillium*. The identification of *Aspergillus* and *Penicillium* species was verified by DNA-sequencing (section 3.6). After re-inoculation the isolates were sorted again based on the

macroscopic characteristics of fungal colonies and from these up to three colonies were identified by DNA-sequencing (section 3.6).

3.6 Molecular identification of cultivated fungal species

Small pieces of mycelia from selected colonies (described in section 3.5) were used for DNA extraction according to Stewart and Via (1993) with some modifications.

Isolates of *Fusarium* spp. were amplified in the translation elongation factor (EF) 1α coding region using primers EF-1 and EF-2 according to O´Donnell *et al.* (1998). Isolates of *Aspergillus* spp. and *Penicillium* spp. were amplified in the β-tubulin (Bt2) gene using primers Bt2a and Bt2b according to Glass and Donaldson (1995).Isolates of unknown species were amplified in the internal transcribed spacer (ITS) region according to Gardes and Bruns (1993) and ITS4 according to White *et al*. (1990). Amplification of DNA and purification of PCR products were done as described in Paper II.

Amplicons of the fungi were sequenced and the GenBank database from NCBI webpage (http://www.ncbi.nlm.nih.gov/BLAST) with the BLASTN algorithm (Altschul *et al.*, 1997) was used to compare sequences.

3.7 Chemical analysis of forages

Dry matter content was determined by drying the samples in two steps; first, samples were dried for 18 hours at 55 °C, weighed after air equilibration and ground in a hammer mill to pass a 1 mm screen, and then dried again for 20 h at 103 °C in a forced air-oven. *In vitro* digestible organic matter was analysed according to Lindgren (1979). Crude protein concentration was measured using the Kjeldahl method (Bremner & Breitenbeck, 1983). Concentration of water-soluble carbohydrates (WSC) was analysed according to Larsson and Bengtsson (1983). Neutral detergent fibre (NDF) concentration was analysed according to Van Soest *et al.* (1991) with the modification of Chai and Udén (1998). Acid detergent fibre (ADF) concentration was analysed according to AOAC (1990; Index no. 973.18). Lignin concentration was analysed using permanganate according to Robertson and Van Soest (1981). Determination of ash concentration was performed by incineration for 3 h at 550 °C.

Sample liquid was used for measurement of pH, concentration of volatile fatty acids (VFA), ethanol, 2,3-butanediol and lactic acid. The liquid was extracted from forage samples and the analysis was performed according to Andersson and Hedlund (1983). Ammonia-N concentration was determined in diluted liquid by direct distillation using Kjeltec Auto System 1020 (FOSS,

Höganäs, Sweden), and a correction was made for the dilution, using the following formula: g/kg N in forage sample = 1.84 x g/kg N in 1:1 diluted samples) – 0.002 (r^2 = 0.95, n = 85) (Ericsson, B. Swedish University of Agricultural Sciences, Uppsala, Sweden; personal communication, 2011).

3.8 Mycotoxin analysis

In Paper III, freeze-dried core samples from 100 of the farms (randomly selected) were used for analysis of mycotoxins. Liquid chromatography mass spectrometry (LC-MS/MS) was used to analyse the mycotoxin concentrations with a multi-mycotoxin method described by Rasmussen *et al*. (2010).

The following eleven mycotoxins were analysed: patulin, deoxynivalenol (DON), nivalenol (NIV) gliotoxin, 3-acetyldeoxynivalenol (3-ACDON), alternariol, T-2 toxin, HT-2 toxin, zearalenone (ZEA), enniatin B (ENN B) and beauvericin (BEAU).

3.9 Production variables

During each farm visit, the forage producer was interviewed using a standardized questionnaire to collect information about production and management of the bales (described in Paper III). The most common bale format sampled in Sweden and Norway (Paper II and Paper III) were big round bales (65 %) followed by medium sized square bales (13%) and big square bales (9%). The remaining proportion contained all different bale sizes in both round and square formats as well as double square bales (Paper III). Almost half (47 %) of the farms used eight layers, 12 % of farms used ten layers, 9 % used 12 layers, 9 % used ≥ 16 layers and 7 % used 14 layers of polyethylene stretch film.

Practically all bales had white plastic polyethylene film (95 %). Bales were mainly stored in the field (65 %) while one-third (36 %) were stored at prepared ground surfaces. On a small number of farms (5 %) bales were stored on wooden pallets. During wilting, the herbage was put in windrows (55 %) or wide-spread (45 %). At majority of the farms (65 %), the forage was fed to horses, but also to cattle (43 %) and sheep and goats (6 %).

3.10 Primer testing for high throughput sequencing

A separate project was performed to test new primers for high throughput sequencing using the 454 sequencing platform. Three samples were included from Paper I, II and III: one fresh herbage sample (from Paper I) and two

wrapped core forage samples (from Paper II and III). Furthermore, artificial samples consisting of eleven fungal species and field samples consisting of soil, wood and wheat roots were included. The herbage and wrapped forage samples were freeze-dried and thereafter DNA was extracted and purified. Three new (fITS7, gITS7 and fITS9) and one previously used (ITS1f) primers together with ITS4 were used to amplify the DNA in the ITS-region. Sequences were analysed using SCATA pipeline and thereafter compared for similarity using BLASTN algorithm. A detailed description of PCR reaction, primers and bioinformatics analysis of the sequences is given in Paper IV.

3.11 Statistical analysis

The statistic packages SAS 9.1, 9.3 and/or 9.4 for Windows (SAS Institute Inc., Cary, NC, USA) were used for all statistical evaluations. Values were deemed significantly different when $P< 0.05$.

3.11.1 Effect of harvest time on microbial composition in herbage and haylage (Paper I)

After testing residuals for normal distribution, analysis of variance using the GLM procedure of SAS was performed. Microbial variables were transformed to log-form to become normally distributed. Values below lower detection limits were set to half the lower detection limit for each particular analysis.

3.11.2 Presence of fungi and fungal species using different sampling methods (Paper II)

The number of fungal species detected with Method I, II and III was compared using the GLM procedure in SAS. Sampling method, incubation temperature and culture media were treated as independent variables. The procedure FREQ and chi-square tests were used in SAS.

3.11.3 Correlations between presence of fungi, presence of mycotoxins, chemical composition and bale production variables (Paper III)

Individual farms were the experimental unit. In the analysis of total fungal presence at farm level, Methods I, II and II were treated separately. Culture media and incubation temperatures with the highest counts of CFU was used as a quantification of fungi in the forage in Method III. The data was handled in

two ways; normally distributed continuous variables (A); and identification of variables important for the presence of fungi (B):

A. Mixed model was used and least square means (LSM) were calculated for continuous variables (chemical composition of forage and specific management variables), $P<0.05$.

B. The procedure LOGISTIC was used to model the odds ratio for presence of fungi using multivariate regression. Qualitative data from Method I and II and quantitative data from Method III was transformed to 1 (presence of fungi) or 0 (absence of fungi). All management and chemical variables were entered into the model, selecting for inclusion at $P<0.05$ using the statement SELECTION=FORWARD.

Correlations between the presence of mycotoxigenic fungi and the presence of mycotoxins that these fungi can produce were calculated using Pearson´s chi-square test and the statement PROC CORR in SAS 9.3. The probability to find mycotoxins was also tested with the procedure PROC LOGISTIC where presence of fungi, chemical composition, and bale management variables were included, $P<0.05$ using the SELECTION=FORWARD statement.

4 Results and discussion

4.1 Microbial composition in fresh herbage and haylage at different forage harvest times (Paper I)

In an experimental study (Paper I), early, moderate or late harvest date of the primary growth resulted in different microbial loads in the fresh herbage and in the haylage. However, these differences were not consistent with increasing delay in harvest date for all types of microorganisms. In the herbage, later harvest date resulted in increased counts of yeast, LAB and enterobacteria. Counts of clostridia were not affected by the harvest date. Counts of filamentous fungi were higher in the herbage harvested in July compared to June, but not compared to August. The increase in yeast and LAB counts with later harvest date was in agreement with results from an experiment using laboratory silos where counts of enterobacteria, yeast and filamentous fungi increased with later harvest dates of primary growth (Müller, 2009).

In total, 15 filamentous fungi species were identified in the herbage. The most predominant fungal species were *Cladosporium cladosporioides* in June, *F. poae* in July, and *Mucor fragilis, F. poae and F. sporotrichioides* in August. The most predominant genera were *Cladosporium* in June, and *Fusarium* in July and August (Figure 3a).

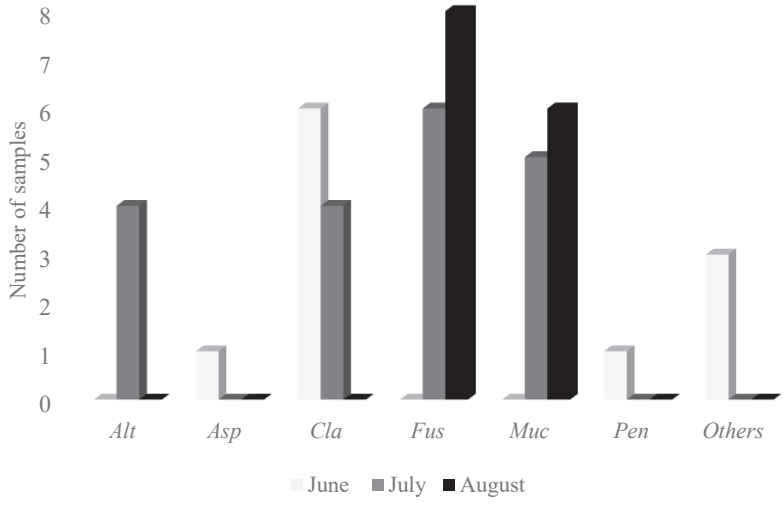

Figure 3a. Fungal genera/order present in samples of herbage from primary growth harvested in June, July and August (N=27), *Alt* (*Alternaria* spp.), *Asp* (*Aspergillus* spp.), *Cla* (*Cladosporoium* spp.), *Fus* (*Fusarium* spp.), (*Penicillium* spp.) and *Others*, and the order *Muc* (*Mucorales*). Bars shown as zero are values below the lower detection limit.

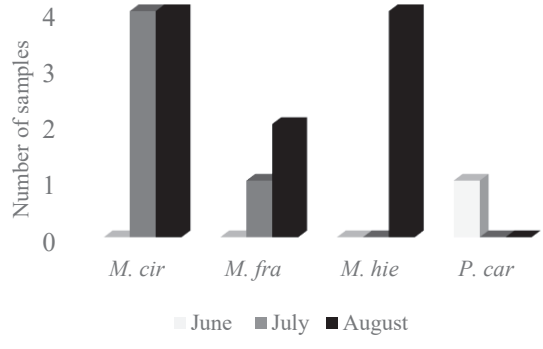

Figure 3b. Fungal species present in samples of haylage from primary growth harvested in June, July and August (N=27), *M. cir (Mucor circinelloides), M. fra (Mucor fragilis*) and, *M hie* (*Mucor hiemalis*). Bars shown as zero are values below the lower detection limit.

For haylage, differences in microbial load were also present but did not reflect the composition in the herbage, with the exception of LAB counts which

increased with later harvest. *Enterobacteria* counts were highest in haylage harvested in August. Counts of yeast were comparable in July and August haylage but lower in June haylage. Clostridial counts in haylage did not differ between the harvest dates. Fungal counts were highest in haylage from August and lowest in June. There were no differences between June and July, or July and August.

In total, four species of filamentous fungi were identified in haylage. In June, *Penicillium carneum* was the only fungal species detected. The most predominant species in July was *Mucor circinelloides* and in August, *Mucor hiemalis* and *M. circinelloides* were most common (Figure 3b). Many of the species (*i.e.* species within the genera *Cladosporium* and *Fusarium*) detected in the herbage were not detected in the haylage. One exception were species within the order *Mucorales* that were present in both herbage and haylage from July and August harvests. Species within *Mucorales* have previously been found in baled silage in Ireland (O'Brien *et al*., 2007) and Norway (Skaar, 1996). In the present study, one species (*P. carneum*) was detected in haylage but not in the herbage (Figure 3b). The differences in the species composition in herbage compared to haylage indicate that a selection of species may take place during haylage preservation. Also, the number of fungal species in haylage increased with advancing harvest date.

4.2 Sampling methods affect detection of fungi in wrapped forages (Paper II)

Different sampling methods for forages bales may lead to different results when determining the presence of fungi for a variety of reasons. In Method I, only visible fungi on the bale surface were sampled. In Method II and III, a representative sample from the entire bale was taken by drilling core samples. In this study, depending on which Method (I, II or III) that was used, the number of fungal species differed (Paper II). When combining the results from all Methods (I, II and III), fungi in bales was found in forages at 89 % of the farms visited and a total of 52 species were detected.

The use of Method II resulted in a higher number of species (47 species) being detected compared to Method I (17 species) and III (26 species) ($P<0.01$). Taking all species isolated using MEA at 25 ºC into account, fungi were detected in bales on 52 % (Method I) of the farms, and using both MEA and DG18 and 25 ºC and 37 ºC, fungi were detected in bales on 77 % (Method II) and 56 % (Method III) of the farms ($P<0.001$).

The most frequently found species in all methods was *P. roqueforti* and when combining all methods this species was found in forages at 48 % of the farms.

When combining two methods (Method I and II, I and III or II and III), *P. roqueforti* was found in forages at 14 to 15 % of the farms, irrespective of which two methods were combined. A similar pattern was seen for *A. fumigatus* (Paper II).

Comparisons of Method I, II and III was performed by using only data from MEA plates at 25 °C. The different methods resulted in different distributions of fungal genera/species/order ($P<0.05$) (Table 1). The most common genera in both Method I and II was *Penicillium* species followed by *Arthrinium*. In Method III, the most frequently occurring genera was *Penicillium* followed by the species *Sordaria fimicola* and (Table 1). These differences could be explained by that *Arthrinium* spp. is a non-sporulating fungus and thus may be underrepresented when only dilution plating is used (Method III) where sporulating species could be overrepresented.

In Method II pieces of plant material is placed directly onto the culture medium without any processing, whereas in Method III, the sample is homogenised in a solution that aids the release of spores into the solution (Samson *et al.*, 2010). *Fusarium* species could also be non-sporulating depending on the type of substrate, incubation temperature and on a light and dark cycle (Samson *et al*., 2010). If these species are of interest, dilution plating should be combined with direct plating or with other methods that do not underestimate the non-sporulating species.

Table 1. *Fungal species/genera/order detected with Method I, Method II or Method III using MEA at 25 ºC for samples of wrapped forage from 124 farms in Sweden and Norway. Numbers are in % (no. of farms in brackets). The distributions of species/genera/order differed between methods at P<0.05.*

Fungal species/genus/family	Method I	Method II	Method III
Arthrinium spp.[a]	18 (22)	31 (39)	11 (14)
Aspergillus spp. (5 species)	8 (10)	5 (6)	2 (3)
Cladosporium spp. (3 species)	2 (3)	6 (7)	6 (7)
Eurotium herbariorum[b]	5 (6)	2 (3)	3 (4)
Fusarium spp. (8 species)	9 (11)	2 (3)	2 (2)
Mucorales (5 species)	16 (20)	15 (19)	8 (10)
Other species (10 species)	1 (1)	10 (12)	4 (5)
Penicillium spp. (16 species)	24 (30)	42 (52)	35 (43)
Sordaria fimicola[b]	6 (7)	22 (27)	31 (38)

[a]Unknown number of species

[b]Only one species within the genus

4.2.1 Presence of visible fungal patches (Method I)

Visible patches of mycelia on bale surfaces were present on over half (52 %) of the farms visited, with a median bale surface area coverage of 1.0 % (minimum 0 %, maximum 8.1% and average 0.4 %) (Figure 4). This indicates that visible fungi was common on the bale surfaces among the sampled farms, even if the surface coverage was comparably small in this study. Other studies have reported larger coverage of visible mycelia on the bale surface of wrapped forages. For example in Ireland, 91 % (58 of 64) of the bales had an average of five percent of the bale surface covered (O'Brien *et al*., 2005a). Another Irish study showed that 92 % (331 of 360) of the bales had visible fungal growth on the bale surface covering on average six percent of the surface (O'Brien *et al*, 2008).

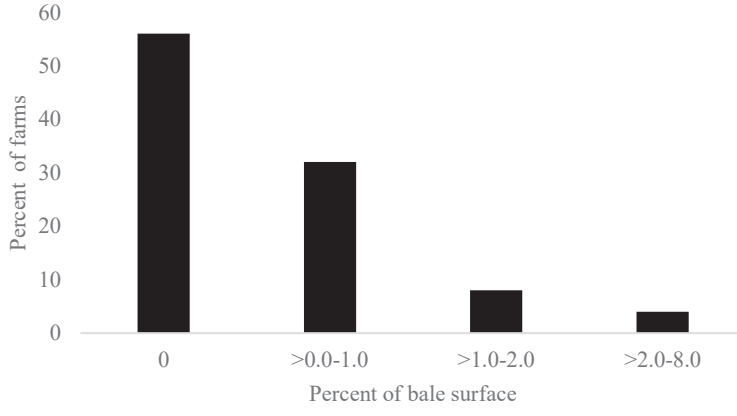

Figure 4. Percentage of farms with visible fungal patches on their bale surfaces and the percentage range of bale surface covered (N=109 farms).

Seventeen fungal species were identified from the visible patches of mycelia on the bale surfaces. The single most common species was *P. roqueforti* (28 % of the farms) followed by species from *Arthrinium* and *A. fumigatus*. Some of the species were spore and mycotoxin producers such as *A. fumigatus* and *P. roqueforti*. The fungus *P. roqueforti* has also been reported to be the most common species found on the surfaces of wrapped bales in Ireland (O'Brien *et al*., 2008; O'Brien *et al*., 2005a) and in Norway (Skaar, 1996).

Variation in colour and appearance (non-sporulating or sporulating) were noted. The colours of the mycelial patches were either white, green or brown. *Arthrinium* spp. were observed as white patches. *Aspergillus* species were observed as white, green or brown. Species within the genus *Mucor* were seen

in white and brown colour. Visible spores were observed with all species of *Penicillium* and *Aspergillus* colonies on bale surface patches. Spores were also observed with *Mucor circinelloides*. It was not possible to characterise the species by ocular inspection of the patches.

The result of this study showed that visible fungi on the bale surface was not a good indicator of fungal presence inside the bale. These result are in agreement with results from an Irish study where bales without visible patches of mycelia fungi were present in drilled core samples (O'Brien *et al.*, 2006a). Furthermore, bales with visible mycelia on their surface had higher fungal CFU in cored samples taken from part of the bale where no mycelia could be observed, compared with bales without any visible mycelia (O'Brien *et al*., 2007). This is important as the hygienic quality of a newly opened bale is based mainly on ocular and olfactory inspection by the person feeding the animals. To discard only the forage with visible fungi from bales with visible fungi on the surface may not be sufficient to reduce animal health risks.

4.2.2 Direct plating of forage (Method II)

With sampling Method II, 47 fungal species were found in bales from 77 % of the farms. The single most frequently occurring genus was *Arthrinium* found on 47 % of the farms. Samples of *Arthrinium* spp. were not further identified by sequencing of other fungal DNA regions as *Arthrinium* spp. mainly are saprobes on grasses (Crous & Groenewald, 2013). *Arthrinium* species have previously been detected in small number of silage samples in Norway (Skaar, 1996). The single most frequent found fungal species with Method II was *P. roqueforti,* which was present in bales at 28 % of the farms, followed by *S. fimicola* found in bales at 25 % of the farms. Most of the detected species within the genus *Penicillium* were detected with Method II (Paper II). Furthermore, Method II detected fungi in bales from the highest number of farms compared to the other two methods. It was also the method that detected the highest number of fungal species. This may be explained by the chance of culturing species that grow with hyphae being higher with direct plating (Method II) compared to dilution plating (Method III) as discussed previously.

4.2.3 Dilution plating and colony-forming units (Method III)

In total, 26 fungal species were found in bales on 56 % of the farms with Method III. The single most frequently occurring fungi in bales using Method III was *P. roqueforti* (28 % of the farms) followed by *Arthrinium* spp. (15 % of the farms),

Aspergillus fumigatus (7 % of the farms) and *Eurotium herbariorum* (7 % of the farms).

National guidelines in Sweden recommends maximum of 5 $\log_{10}$ CFU of filamentous fungi per gram of feed for ruminants (Spörndly, 2003) and equines (Jansson *et al*., 2013). In this study, 21 farms had forages that exceeded 5 $\log_{10}$ fungal CFU per gram of forage. On those 21 farms, *Penicillium* species were present in bales on 18 farms and *Aspergillus* species on four farms, togheter with other fungal species. On some farms, none of the genera with the ability to produce spores or mycotoxins were present. This indicate that only fungal counts with no identification of species is of less value for evaluation if the forage is appropriate as an animal feed.

4.3 Culture media and incubation temperature (Paper II)

In Method II and III, the combination of two culture media (*i.e.*, MEA and DG18) and two incubation temperatures (*i.e.*, 25 and 37 °C) were used to detect the presence of and to enumerate fungi in samples of wrapped forage.

Culture media did not affect the outcome of which fungal genera/order that were detected in Method II and III, except for *Mucorales* that was detected in higher frequency on MEA compared to DG18 ($P<0.05$). In Method II and III, the most frequently isolated genera/species on MEA, irrespective of incubation temperature, was *Arthrinium* spp. followed by *P. roqueforti* and *S. fimicola*. On DG18, *Arthrinium* spp. was the most frequently isolated fungus followed by *Eurotium herbariorum* and *P. roqueforti*.

Incubation temperature influenced the outcome of fungal cultivation (Table 2). In Method II and III, the most frequently occurring species/genera at 25 °C, irrespective of culture media, was *P. roqueforti* followed by *Arthrinium* spp. and *S. fimicola*. The most frequently occurring species/genera at 37 °C was *Arthrinium* spp. followed by *A. fumigatus* and *S. fimicola*. Several fungal species/genera were found more often at 25 °C compared to 37 °C such as *Penicillium* spp. ($P<0.001$), *P. roqueforti* ($P<0.001$) and *Fusarium* spp. ($P<0.05$). *Aspergillus* spp. ($P<0.05$) and *A. fumigatus* ($P<0.05$) were the only genera/species that were found more often at 37 °C compared to 25 °C (Paper II). Thus, incubation at both 25 and 37 °C was necessary to detect as many species as possible in samples of wrapped forage (Paper II).

As both *A. fumigatus* and *P. roqueforti* are fungi of interest due to their capabilities of spore production and mycotoxigenic properties, it is important to choose culture media and incubation temperatures that will make it possible to detect these species in samples of wrapped forage, as their presence may pose an animal health risk.

Table 2. *Mycotoxigenic fungal species*[a] *cultivated with direct plating using two culture media (malt extract agar (MEA) and dichloran glycerol agar (DG18)), and two incubation temperatures (25 and 37 °C), from haylage samples from 124 Swedish and Norwegian farms. Numbers are in % (no. of farms in brackets)*

Substrate:	MEA	MEA	DG18	DG18
Temperature:	25 ºC	37 ºC	25 ºC	37 ºC
Alternaria alternate	1 (1)	N.D.	2 (2)	1 (1)
Aspergillus candidus	N.D.	N.D.	1 (1)	N.D.
Aspergillus flavus	N.D.	1 (1)	N.D.	N.D.
Aspergillus fumigatus	3 (4)	4 (5)	N.D.	6 (7)
Aspergillus niger	1 (1)	N.D.	1 (1)	3 (3)
Aspergillus versicolor	1 (1)	N.D.	N.D.	N.D.
Byssochlamys nivea	2 (2)	N.D.	2 (2)	N.D.
Chaetomium globosum	1 (1)	N.D.	N.D.	N.D.
Eurotium herbariorum	3 (4)	2 (2)	11 (14)	4 (5)
Fusarium avenaceum	N.D.	N.D.	2 (2)	N.D.
Fusarium culmorum	N.D.	N.D.	1 (1).	N.D.
Fusarium equiseti	N.D.	N.D.	2 (2)	N.D.
Fusarium graminearum	1 (1)	N.D.	1 (1)	N.D.
Fusarium oxysporum	N.D.	N.D.	2 (2)	N.D.
Fusarium poae	2 (2)	N.D.	1 (1)	1 (1)
Fusarium sporotrichioides	N.D.	N.D.	2 (2)	N.D.
Fusarium verticillioides	N.D.	N.D.	1 (1)	N.D.
Paecilomyces variotii	N.D.	1 (1)	N.D.	N.D.
Penicillium aurantiogriseum	1 (1)	N.D.	N.D.	N.D.
Penicillium brevicompactum	2 (2)	N.D.	N.D.	N.D.
Penicillium carneum	2 (3)	N.D.	N.D.	N.D.
Penicillium citrinum	1 (1)	N.D.	N.D.	N.D.
Penicillium commune	1 (1)	N.D.	N.D.	N.D.
Penicillium corylophilum	2 (3)	N.D.	N.D.	N.D.
Penicillium crustosum	1 (1)	N.D.	N.D.	N.D.
Penicillium echinulatum	2 (2)	N.D.	N.D.	N.D.
Penicillium expansum	1 (1)	N.D.	1 (1)	N.D.
Penicillium fumiculosum	N.D.	1 (1)	N.D.	N.D.
Penicillium glabrum	2 (3)	N.D.	N.D.	N.D.
Penicillium olsonii	1 (1)	N.D.	N.D.	N.D.
Penicillium paneum	4 (5)	N.D.	N.D.	N.D.
Penicillium roqueforti	22 (27)	2 (2)	16 (20)	1 (1)
Penicillium verrucosum	1 (1)	N.D.	N.D.	N.D.

[a]Mycotoxin producing fungal species according to Samson *et al*. (2010)

N.D., not detected.

4.4 Occurrence of mycotoxins (Paper III)

Forage samples were analysed for eleven mycotoxins, of which nine were detected. One or more of these nine mycotoxins were present in bales on 39 %

of the 100 farms that were randomly selected for testing. The most frequently detected mycotoxins were ENN-B (14 % of the samples), DON (12 % of the samples) and BEAU (10 % of the samples) (Table 3). Patulin and NIV concentrations were below the lower limit of detection in all tested samples.

Table 3. *Concentration of mycotoxins in haylage samples from 124 Swedish and Norwegian farms, number of samples were mycotoxins were detected and recommended maximum concentrations in feeds by the EU Commission (see table footnot[b, c]). Mean, minimum and maximum values calculated only from samples where the mycotoxin was detected*

Mycotoxin µg per kg air-dry sample	Mean (µg)	Minimum (µg)	Maximum (µg)	Number of samples above detection limit	Recommended maximum concentration (µg per kg feed)[a]
3-acetyldeoxynivalenol (3-ACDON)	179	70	288	2	N.A
Alternariol	212	11	1452	8	N.A
Beauvericin (BEAU)	248	11	988	10	N.A.
Deoxynivalenol (DON)	238	69	479	12	8000[b]
Enniatin B (ENN-B)	56	10	283	14	N.A.
Gliotoxin	51	44	57	2	N.A.
HT-2 toxin	35	19	78	4	2000[c]
T-2 toxin	9	8	11	3	2000[c]
Zearalenone (ZEA)	8	8	8	1	2000[b]

[a]Moisture content of 12 %
[b]Guidance values from EU Commission (2006) for feed materials for cereals and cereal products with the exception of maize by-products
[c] Guidance values from EU Commission (2013) for unprocessed cereals (wheat, rye and other cereals except barley and oats)
N.A.=Not available

The concentration of DON and ZEA were below the European Commission guidance values (Table 3). In another study the maximum concentration of DON and ZEA in grass silages were 167 and 67 µg per kg, respectively (Skladanka *et al*., 2013), however there are few reports on presence of *Fusarium* toxins in grass silage (Panasiuk *et al.*, 2019).

There are no EU Commission recommendation for the maximum limits of BEAU and ENN-B in feeds due to insufficient information on their toxicity in mammals including humans (EFSA, 2014). The European Food Safety Authority (EFSA) has concluded that acute intoxication of BEAU and ENN-B are unlikely for farm animals (EFSA, 2004). In the current study (Paper III), the mean concentration of BEAU was low (248 µg per kg) compared to concentrations found in feeds sourced from Korea (720 µg per kg) (Kyung-Eun *et al*., 2010). The mean concentration of ENN-B was also low (56 µg per kg), compared to values recorded in European study where concentrations ranged from <0.3 to 1514 µg per kg in feed samples (Fraeyman *et al.,* 2017). In general,

BEAU and enniatins are regarded as contaminants of grain and grain-based products (Fraeyman *et al*., 2017). Ambient temperature and precipitation during the growing period of the grain crop have been found to be the most important factors for both the presence and concentrations of enniatins and BEAU in wheat (Staneiu *et al*., 2017). If this is valid also for forage crops is not known. The results of this study indicates that in this study, the presence of BEAU and ENN-B may require further attention if these toxins are suspected to cause health problems in forage eating lifestock in the future.

Gliotoxin, was detected in drilled forage samples from two farms only in this study (Table 3). In two other studies of maize silage gliotoxin concentrations were 6.6 to 11.9 µg per kg (Richard *et al.*, 2009) and 5.1 to 6.5 µg per kg (Pereyra *et al.*, 2008), which was about one tenth to one fifth of the average concentration found in the present study (Table 3). Gliotoxin can have immunosuppressive, antimicrobial, apoptotic and cytotoxic effects on animals but the health effects on ruminants and horses are not well documented (Morgavi *et al*., 2004).

In the present study, alternariol was found in comparably high concentrations (maximum and mean 1452 and 212 µg per kg, respectively) (Table 3). In a study of wheat more than 95 % of the samples contained more than one type of toxins from *Alternaria* species (Xu *et al*., 2016). The average alternariol concentration in that study was 7.9 µg per kg compared to 212 µg per kg in the present study (Table 3). The concentrations in most grain samples have been reported to be less than 100 µg per kg with an average of 221 µg per kg (Fraeyman *et al*., 2017). Alternariol is cytotoxic and genotoxic to bacteria but *in vivo* toxicity studies in animals are limited (Fraeyman *et al.,* 2017). For toxicological reasons, a threshold of 2.5 ng alternariol per kg body weight and day at mean chronic dietary exposures for humans have been established by the EFSA (2011a). The threshold value was thereafter evaluated by the EFSA panel on Contaminants in the food chain (CONTAM panel) but studies up until 2014 have shown inconsistent results. The European Commission therefore have still no recommended maximum values for toxins from *Alternaria* species in food and feed in Europe (EFSA, 2016).

The mean concentration of HT-2 and T-2 were 35 and 8 µg per kg, respectively, in the current study. Both toxins were below the European Commission guidance value of 2000 µg per kg (Table 3). The presence of 2 mg of T-2 toxin per kg of mouldy dry maize has been reported to cause gastroenteritis in lactating cows (Hsu *et al*., 1972). Other toxins could also been present in the mouldy feed, making it difficult to establish any cause and effect of T-2 toxin (Hsu *et al*., 1972). The concentrations of HT-2 and T-2 toxins have previously been reported in maize silages in Switzerland with maximum values of 84 and 130 µg per kg, respectively (Eckard *et al*., 2011). This was

considerably higher EFSA (2011b) concluded that these toxins are more prevalent in cereals compared to wrapped forages.

The toxin 3-ACDON was detected in two samples and with a mean concentration of 179 µg per kg (Table 3). This result is similar to results from other studies where concentration of acetyl-deoxynivalenol (AcDON) (sum of toxins 3-ACDON and 15-acetyldeoxynivalenol (15-ACDON)) in two of nineteen samples was on average 218 µg per kg (Eckard *et al*., 2011). The EFSA concluded that the reported dietary concentrations of 3-ACDON (and sum of DON, 15-ACDON and DON-3-glucoside) found in feed are unlikely to cause health problems in horses and ruminants (EFSA, 2017). The European Commission have at present no guidance value of the maximum limits of 3-ACDON in feeds.

4.5 Associations between the presence of fungi, mycotoxins, forage chemical composition and forage management variables (Paper III)

The chemical composition of the forage samples from the 124 farms varied considerably and indicated that harvests in very different plant maturity stages were included (Table 4). The DM content was on average 627 g per kg, indicating a comparably dry forage (Table 4).

Table 4. *Chemical composition of forage samples from wrapped bales at 124 farms in Sweden and Norway. Composition given in g per kg DM if not otherwise mentioned (Paper III)*

Variable	Mean	Minimum	Maximum	Median
Dry matter, g per kg	627	279	884	653
Ash	69	37	132	67
Crude protein	105	55	271	96
In vitro digestible organic matter	782	642	921	781
Neutral detergent fibre	562	400	672	570
Acetic acid	3.8	0.3	33	2.0
Lactic acid	14	1.8	74	6.7
Ethanol	7.0	0.2	42	5.8
pH	5.3	4.2	6.1	5.5

The forage producer's response from the survey about forage production and bale management characteristics were used to model the odds ratio of presence of fungi and/or mycotoxins using multivariate regression. Dry matter content, pH, seal integrity, number of film layers, wilting technique, harvest number, latitude and year were all factors that were associated with increased or

decreased risk of fungal presence (Table 5). All factors were however not influential in all three methods for detection of fungi.

Table 5. *Effect of management factors and chemical composition of wrapped forages on risk of presence of filamentous fungi isolated with Method I, II and III*

Type of variable	Significant with sampling method	Direction of effect
Dry matter	I, II, III	Higher risk of fungal presence at higher dry matter contents.
Harvest no	II, III	Higher risk of fungal presence in primary growth harvests compared to regrowth harvest.
Year	I, III	Higher risk of fungal presence in 2010 compared to 2011.
Layers of polyethylene stretch film	III	Higher risk of fungal presence with <8 layers of polyethylene stretch film.
Seal integrity	I	Higher risk of fungal presence at lower seal integrity.
Wilting	I	Higher risk of fungal presence when wilting grass in windrows compared to wide-spread.
Acetic acid	III	Higher risk of fungal presence at higher acetic acid concentration.
Ethanol	III	Higher risk of fungal presence at higher ethanol concentration.
pH	I	Higher risk of fungal presence at higher pH.
Latitude	I	Higher risk of fungal presence at higher latitudes.

The only variable that entered the final model for all three sampling methods was DM content, where increasing DM content was associated with higher a risk of fungal presence irrespective of which method was used for isolation of fungi. Harvest number and sampling year entered the final model in two of the

sampling methods (Table 5), where the risk of fungal presence was higher in primary growth harvests compared to regrowth harvests with Methods II and III, and the risk of fungal presence was higher in sampling year 2010 compared to 2011 in Methods I and III. Seal integrity, wilting, acetic acid, ethanol, pH and latitude entered the final model in one of sampling Methods I and III (Table 5).

4.5.1 Dry matter and pH

Dry matter content varied between 229 to 884 g DM per kg (Table 5). In this study increasing DM contents was associated with increased risk of fungal presence in the forage (Table 5). Increasing DM content was also associated with increasing pH (r=0.78, P>0.001). O'Brien *et al*. (2007) reported that increasing pH and DM content were positively correlated with an increased presence of fungi in wrapped bales in Ireland. Consequently, forage with lower DM content is at lower risk of occurrence of filamentous fungi, not only in a DM contents of 157 to 665 g per kg (mean 349 g DM per kg) as reported by O'Brien *et al.* (2007) but also in the DM range of 229 to 884 g per kg (mean 653 g DM per kg), as shown in the present study. Furthermore, the higher risk of finding visible fungal patches on the bale surface was higher with a higher pH.

4.5.2 Harvest number

Most of the sampled bales were harvested from the primary growth (73 %), whereas the remaining proportion (27 %) consequently were regrowth harvests. The risk of fungal presence in bales from primary growth harvest was higher compared to in regrowth harvest bales (Table 5). However, primary growth harvests also generally had higher (P<0.01) DM content (650 g per kg) compared to the regrowth harvests (560 g per kg). This may explain the higher risk of fungal presence in forage from the primary growth harvests as the risk of fungal growth increased with increasing DM content (Table 5). In addition, the physical structure of the grass may differ between primary and regrowth harvests as most grasses used for forage production in Sweden and Norway regrow with mainly leaves which may be less prone to puncture the stretch film, and thereby affect seal integrity, compared to the stems in the primary growth. However, the seal integrity was higher in the primary growth than in regrowth harvests (220 s and 98 s respectively, P<0.05).

Another factor contributing to the higher risk of fungal presence in the primary growth harvests could be that bales from this harvest were produced earlier in the summer season and exposed to high temperature variations due to sun radiation during summer storage. This may lead to considerable differences

in air pressure leading to gas exchange through the stretch film layers also when the seal integrity is high. Bales produced later in the season will be stored under less fluctuating and in general lower temperatures, which may have a restrictive effect on fungal growth in the bales.

4.5.3 Year

The risk of fungal presence was higher during the first sampling year compared to the second. Higher risk of founding fungi first sampling year could be explained by a higher mean temperature during the sampling period in 2010 (11.1 ºC) compared to sampling period in 2011 (7.0 ºC) according to meteorological data (SMHI, 2018). Also, the sampling started later in the first year (April) compared to in the second year (February). During the spring, ambient outdoor temperature increases over time meaning that bales from the first sampling year was subjected to higher temperature over longer time compared to bales from the second sampling year. This may have had an influence on fungal growth, especially if seal integrity has been compromised.

4.5.4 Layers of polyethylene stretch film

In this study, presence of visible fungal patches on bale surfaces (Method I) and the number of plastic polyethylene film layers were not found to be related. However, lower seal integrity increased the risk of visible fungal patches on bale surfaces (Table 5). It has previously been shown that seal integrity and CO_2 content was higher when eight layers of stretch film was used compared to four and six layers, as reviewed by Spörndly *et al*. (2017).

O'Brien *et al*. (2007) reported that the number of fungal patches on surfaces of baled silage could be reduced if the number of stretch film layers were increased from four to six. In a study where six, eight and ten layers of polyethylene stretch film on small square bales were compared the proportion of CO_2 inside the bales was higher with ten layers compared six layers, but no effect on fungal counts was observed (Müller, 2005).

For baled silage, recommendations has been given by Keles *et al.* (2009) that at least four layers of polyethylene stretch film is needed to achieve an anaerobic environment inside the bales. However, Keles *et al*. (2009) used forage with a lower DM content and forage harvested at an earlier plant maturity, which most likely differed in physical structure, chemical composition and fermentation potential compared to the wrapped forages in this study. The silages described by Keles *et al*. (2009) could be expected to tolerate lower seal integrity better

primarily due to higher compaction of the bales which prevent infiltration of oxygen rich air (Williams, 1994), compared to the forage in the current study.

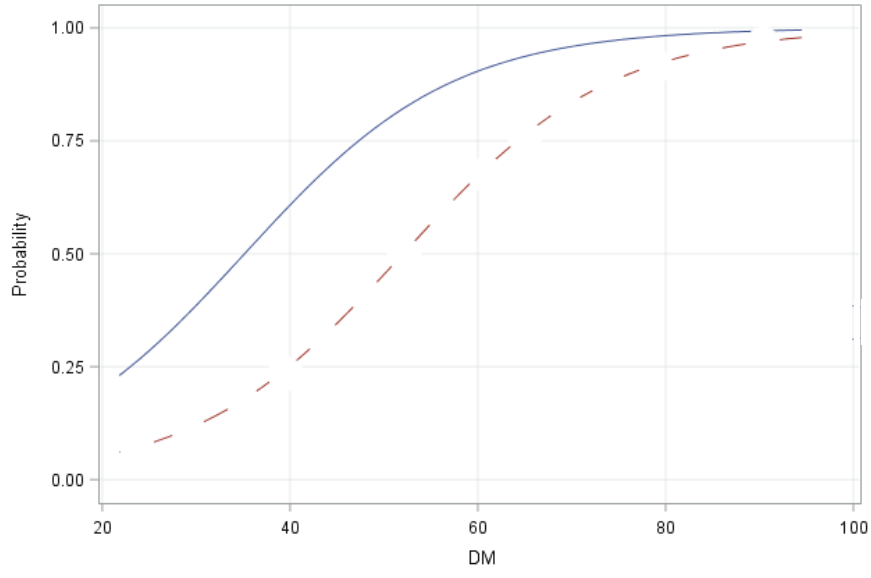

Figure 5. Predicted probability of presence of fungi (Method II) if more (dashed line) or less or equal (solid line) than eight layers of polyethylene stretch film was used in relation to dry matter (DM) contents of the forage.

In the present study, the probability of fungal presence (using sampling Method II) increased with increasing DM contents, and the probability was higher when eight or less layers of stretch film was used (Figure 5). The risk of fungal presence (using Method III) was over five times higher in bales where less than eight layers of stretch film had been applied, compared to when eight or more layers of stretch film had been used (Paper III).

4.5.5 Seal integrity

Seal integrity (tightness) of the bales is an important factor in restricting fungal growth (McDonald *et al*., 1991). About half of the bales tested for seal integrity of the polyethylene stretch film managed to maintain the pressure applied for more than 100 s. Ten percent of the bales were considered as insufficiently tight as the applied negative pressure was kept for < 10 s. The remaining 40 % of the bales had a seal integrity between 10 to 100 s.

Integrity times below 100 s are regarded as insufficiently tight for commercial trade in Sweden. Normal seal integrity time for big round bales with undamaged polyethylene stretch film should be 300 to 1300 s (Spörndly *et al.*, 2008a). In this study, there were fewer bales with seal integrities over 100 s that had growth of visible patches present on their surfaces (Method I) compared to bales with seal integrities of ≤ 100 s. This indicated that seal integrity (tightness) of bales is an important management factor to decrease the risk of fungal presence on the bale surface.

4.5.6 Acetic acid and ethanol

Concentrations of acetic acid and ethanol were similar to previously reported concentrations in wrapped forages with high DM content (Müller, 2015). Higher acetic acid or higher ethanol concentrations were associated with higher risk of fungal presence detected with sampling Method III. However, in other studies, no association between presence of acetic acid and presence of fungi or counts has been reported (O'Brien *et al*, 2007; Skaar, 1996).

The association between higher concentration of acetic acid and the presence of fungi could be regarded contradictive, as acetic acid is known to restrict growth of fungi (Vivier *et al*., 1992). However, the restrictive effect of acetic acid on fungi is dependent on low pH (Woolford, 1975). In the present study, pH was generally higher compared to the pH reported by Vivier *et al*. (1992).

4.5.7 Latitude

With the use of sampling Method I, the risk of fungal presence on bales was greater on farms located at a higher latitude. This indicates that bales produced in the northern part of Sweden and Norway more frequently had visible fungal growth on the bale surface compared to the southern regions. In present study no correlations between fungal presence and DM content or seal integrity was detected.

Increased risk of fungal presence in forage samples from higher northern latitudes was also reported in Ireland, where the numbers of visible fungal patches on the bale surface increased with length of feeding season as farmers at northern latitudes normally have shorter grazing period and longer winter feeding period (O'Brien *et al.*, 2007).

4.5.8 Wilting

Thirty-nine percent of the farmers wilted the herbage for two days before baling and wrapping, while 23 % of farmers wilted the crop for three days. The

remaining proportion of farmers wilted the forage for three, four or more than four days (Paper III). The wilting time in this study had no effect on fungal occurrence in the baled forage. Nevertheless, wilting time has previously been reported as an important factor affecting fungal occurrence. For example, O'Brien *et al.* (2008) reported that forage that was wilted for more than three days was more prone to fungal occurrence compared to forage that was wilted for less than one day. Results from a Norwegian study showed that wilting periods of up to 24 h compared to no wilting decreased fungal counts in silage bales (Skaar, 1996). When evaluating the effect of wilting on the presence of fungi in wrapped forages, it is necessary to differentiate between wilting time in hours (which may be prolonged due to moist weather conditions) and wilting time that results in forage having an increased DM content, as these may differ widely.

Wilting can be performed in different ways, and in this study fifty-five percent of the farms widespread the grass during wilting and 45 % wilted the grass in windrows.

The risk of presence of visible fungal patches on the bale surface (Method I) was higher when wilting the herbage in windrows compared to wide-spreading. Wide-spreading leads to faster drying as the herbage is more exposed to the sun in comparison to wilting in windrows (Spörndly *et al*., 2008b), and this may explain the lower risk of fungal presence in forage that has been wilted wide-spread.

4.5.9 Mycotoxins

In the present study, the presence of fungi detected with each of the three Sampling Methods (I, II and III) was not associated with the presence of mycotoxins. This is not surprising as the presence of a mycotoxin may be the result of previous active fungal growth in the field, and thereby not necessarily still active at the time of sampling. In the present study a mycotoxin and the fungal species that produces that mycotoxin were seldom found in the same sample, except for some cases for gliotoxin, alternariol, HT-2 toxin and BEAU. The toxin patulin was not detected in any of the samples of wrapped forage even though the fungi *P. roqueforti* were present in high frequency. However, not all strains of *P. roqueforti* are patulin producers (Nielsen *et al*., 2006).

There were no correlations between presence of mycotoxin and management factors or forage chemical composition, except for DM content. Wrapped forage samples containing *Fusarium* spp. toxins (BEAU, DON, ENN-B, NIV, HT-2, T2 or ZEA) over the detection limit tended to be dryer compared to samples not containing these toxins (675 vs 615 g DM per kg, $P<0.0642$). Variables

describing silage chemical composition have previously been shown to be unreliable predictors of mycotoxin occurrence (McElhinney *et al.,* 2016).

The presence of mycotoxins in core samples was not correlated with the presence of visible fungal growth on the bale surface (Method I). However, the risk of finding mycotoxins increased when fungal counts in core samples increased (Method III). This result showed that absence or presence of visible fungi on the bale surface was not an indicator of mycotoxin presence or absence in the forage. Another reason for not finding any correlation between mycotoxin and their forming fungal species may be that the detected mycotoxins were present already in the fresh crop before harvest, meaning that mycotoxin and fungal presence on the bale surface have separate origin or causes and are therefore not correlated. As the most frequently found mycotoxin were *Fusarium* derived, and as *Fusarium* are typical field-fungi, the latter explanation seems likely. Lack of correlation between presence of fungi and presence of mycotoxins has also been reported for hay (Raymond *et al*., 2000).

4.6 Other methods of detecting fungi: Primer testing for next generation sequencing (Paper IV)

A next generation sequencing method to detect fungi is through the amplification of the entire ITS region (*e.g.* using primer ITS1f with ITS4 (Begerow *et al*., 2010)). However the traditionally used primer ITS1f may discriminate against fungal species with long amplicons, and consequently development of new primers is required.

The three new primers in the present study, namely fITS7, gITS7 and fITS9, have shorter amplicons that leads to higher efficiency in sequence amplification. Short amplicons decreases the number of necessary PCR cycles compared to the traditionally used primer, ITS1f, and thereby the bias of the fungal community in the sample will be reduced. A degeneration in a single position in primer gITS7 resulted in a high yield of amplification of plant DNA which may disfavour sequencing of fungal DNA. Furthermore, primer fITS7, excluded most species of the genera *Penicillium* and *Mucorales* which are common cultured fungi found in baled forage. The design of primers for amplifications of the fungal kingdom is challenging, *e.g.* the sensitivity of primer mismatches and amplification of nonfungal templates may be difficult to overcome.

This study showed that large-scale sequencing molecular methods could be challenging to use for analysis of the fungal community in samples of wrapped forage. It is important to take into consideration, that within the fungal genera it is not always possible to identify all species such as species within the genera *Aspergillus*, *Fusarium* and *Penicillium*, when using sequence data from the ITS-

region. At present, *Aspergillus* and *Penicillium* species can be identified from a partial sequence of the β-tubulin gene (Glass & Donaldson, 1995), for the identification of *Fusarium* species the translation elongation factor 1α coding region is used (O´Donnell *et al*., 1998). However, when this study was conducted there were no primers accessible for 454 sequencing for other fungal regions than ITS.

In the future, more primers for other regions than ITS need to be developed to cover all possible fungal species that could be present in the wrapped forage. Another factor to take into consideration is that DNA is a stable material and when performing PCR on DNA, dead material could be amplified. This means that fungi that are not viable could be represented in the sequencing result. On the other hand it can be meaningful to detect non-viable fungal species/genera perhaps to achieve better correlations between fungi and mycotoxin presence.

5 Conclusions

The research findings included in this thesis have contributed to improve our knowledge of the types of filamentous fungi present in wrapped forages with high dry matter contents. The following specific conclusions can be drawn:

- fungal species present in herbage differ from fungal species present in the haylage, which indicates a selection of species during haylage preservation,
- direct plating of forage samples results in higher number of fungal species compared to dilution plating,
- the risk of presence of fungi and mycotoxin in wrapped forage was higher with increasing DM contents,
- the risk of fungal presence in forage samples was higher if less than eight layers of polyethylene stretch film was used,
- the risk of mycotoxin presence was higher in bales with higher fungal counts, and
- no management factors or forage chemical composition variables were correlated with presence of mycotoxin,
- large-scale sequencing molecular methods could be useful for analysis of fungal communities in forages but further development of primers is required.

A general conclusion, based on the results from studies presented in this thesis, is that the probability of finding fungi in wrapped forage with DM content between 600 to 800 g per kg was over approximately 0.65 even if more than eight layers of polyethylene stretch film was used. Also, for detection of as many fungal species as possible, direct plating of forage material on more than one substrate at more than one incubation temperature is advisable.

6 Future perspectives

Fungi that could affect animal health negatively are mainly spore and/or mycotoxin producers. Many of these are found within the *Aspergillus*, *Alternaria*, *Fusarium* and *Penicillium* genera, and these were detected in wrapped bales in this study. The single most common species was *P. roqueforti* which can produce several mycotoxins *e.g*. roquefortine C, PR-toxin, patulin and mycophenolic acid (Samson *et al*., 2010; McElhinney *et al*, 2016). When planning the current study we did not include roquefortine C and aflatoxin in the mycotoxin analysis. However, it would have been interesting to see if other *Penicillium* toxins or *Aspergillus* toxins were present in the wrapped forages sampled in in present study since species capable of producing these toxins were present. In future studies of mycotoxins in forages, modern multi-mycotoxin methods that includes a large number of different mycotoxins are of interest.

The 454-sequencing method takes time and is costly, and it is challenging to handle the sequence data. Therefore it is not used anymore. This study (Paper IV) was conducted during year 2010 to 2013 and currently new HTS methods for identification of fungi are available. At present, for example PacBio RSII and Sequel (Pacific Biosciences) are available. Both are third generation HTS platform ideal for sequencing of short to medium lengths amplicons such as the whole sequencing length of the ITS-region of different fungal species/genera (Nilsson *et al*., 2019). The primers described in Paper IV are now used with the third generation HTS-platform. These new methods are used regularly for determining the composition of complex fungal communities and they may also be used in the future for fungal communities in wrapped forages.

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Populärvetenskaplig sammanfattning

Förekomst av mögel i inplastat grovfoder i Norge och Sverige

Idag är hösilage ett vanligt foder till nötkreatur och häst i Norge och Sverige. Hösilage är inplastat vallfoder med en torrsubstanshalt (ts-halt) mellan 50 och 84 % och konserveras genom en kombination av torkning och lufttät lagring. Det skiljer sig mot blötare ensilage som konserveras via mjölksyrajäsning och lufttät lagring. I hösilage sker en svag eller ingen mjölksyrajäsning då mängden tillgängligt vatten är alltför liten för de mjölksyrabakterier som utför själva ensileringen. Det betyder att såväl mikrobiologisk som kemisk sammansättning skiljer sig åt mellan ensilage och hösilage. Produktion av hösilage har många fördelar i jämförelse med höproduktion, det är t ex inte lika väderkänsligt, och inplastade hösilagebalar kan till skillnad från hö lagras utomhus. Det kan vara svårt att lagra hö tillräckligt torrt under vinterhalvåret på våra breddgrader, eftersom hö tar upp fukt ur den omgivande luften. Det ökar risken för mögeltillväxt i höet.

Det kan finnas risk för tillväxt av mögel i inplastat grovfoder också särskilt om syre finns tillgängligt. Syre kan komma in i en inplastad bal på flera sätt, t. ex. genom otät inplastning. Eftersom mögel kan bilda skadliga sporer och/eller gifter (mykotoxiner) är det viktigt att undvika att mögel tillväxer. Med anledning av detta har en studie utförts där syftet var att kartlägga förekomst av mögel och vilka mögelarter som finns i inplastat vallfoder. Syftet var också att undersöka om olika produktionsfaktorer i vallskörden (inklusive fodrets näringsinnehåll) var associerade med ökad risk för förekomst av mögel och mykotoxiner.

I den första studien togs prover från grönmassan precis innan pressning och inplastning av rundbalar vid tre skördetillfällen under sommaren (juni, juli och augusti). Alla skördetillfällen var i förstaskörden. Prover på det färdiga hösilaget togs från de inplastade balarna med hjälp av en ensilageborr. Den mikrobiella sammansättningen jämfördes därefter i grönmasse- och hösilageproverna.

Mängden mögel var högre i grönmassan i juli i jämförelse med juni, men inte i jämförelse med augusti. Mängden mögel var högst i hösilage från augusti och lägst i juni. Det fanns inga skillnader mellan juni och juli, eller juli och augusti.

De mögelarter som påvisades i hösilageproverna var inte desamma som påvisades i grönmasseproverna, och antalet mögel var lägre i hösilaget. Detta kan indikera att det under konserveringen av hösilage sker en selektion av vissa mögelarter.

I studie två samlades prover från inplastat grovfoder med hög ts-halt in under två års tid från 124 gårdar varav 25 var norska och 99 var svenska. På varje gård togs borrprover från tre balar. Plasten avlägsnades från en av balarna och om det fanns synligt mögel på balytan registrerades och provtogs det. Samtidigt som provtagningen inhämtades uppgifter om produktion och lagring av fodret från producenten. Mögelförekomsten analyserades med hjälp av tre olika metoder: med Metod I odlades prover av synligt mycel från balytan på agarplattor; med Metod II placerades små bitar från det borrade provet på agarplattor för uppodling av mögel (direktutlägg); och med Metod III gjordes en spädningsserie av borrprovet som sedan odlades på agarplattor för att kunna göra en bestämning av mängden mögel i provet. Identifieringen av vilka arter som växte på agarplattorna utfördes med hjälp av odling på selektiva substrat och från strukturer synliga i mikroskop, samt med DNA-sekvensering. Borrproverna användes också för analys av mykotoxininnehåll.

Resultatet från studien visade att på fler än hälften (52 %) av gårdarna fanns synliga mögelkolonier på balens yta (Metod I). Totalt 52 olika mögelarter detekterades. Den vanligaste arten i Metod I var *Penicillium roqueforti* som hittades på 20 % av gårdarna. Andra arter som hittades på balens yta var *Artrhinium* spp. (18 %), *Aspergillus fumigatus* (6 %) och *Fusarium poae* (6 %). I Metod II där direktutlägg av borrprov utfördes hittades mögel på 79 % av gårdarna. Vanligast i Metod II var *Artrhinium* spp. (47 %) följt av *P. roqueforti* (28 %) och *Sordaria fimicola* (25 %). I Metod III där en spädningsserie av borrprovet utfördes hittades mögel på 56 % av gårdarna. Den vanligaste mögelarten i Metod III var *P. roqueforti* (28 %) följt av *Arthrinium spp.* (15 %), *A. fumigatus* (7 %) och *Eurotium herbariorum* (7 %).

Studien visade att mögelförekomst var vanlig i balar som provtogs på de utvalda gårdarna. Ökad risk för mögeltillväxt i det inplastade grovfodret berodde på flera faktorer. Användes färre än åtta lager plast ökade risken för förekomst av mögel, medan mer än åtta lager plast minskade risken för förekomst. Sannolikheten att påträffa mögel i en bal var över 0.65 när fodrets ts-halt var från ca 60 till ca 80 %, oavsett hur många antal lager plast som används. Sämre täthet var också en faktor som ökade risken för förekomst av synligt mögel på balarnas yta. Tidigare skörd och bredspridning av grönmassan ledde till minskad

risk. Vid bredspridning torkar grödan fortare och jämnare i jämförelse med strängläggning och därmed minskar förmodligen risken för mögeltillväxt.

Totalt analyserades elva mykotoxiner i 100 slumpmässigt utvalda prover från Norge och Sverige. Nio av dessa påvisades i 39 % av proverna. De tre mest vanligt förekommande mykotoxinerna var enniatin B (14 % av proverna), deoxynivalenol (12 % av proverna) och beauvercin (10 % av proverna).

I den tredje studien testades tre nya primers för svampgenen ITS (internal transcriped spacer) för så kallad 454-sekvensering, en pyrosekvenseringsmetod. Först extraherades DNA direkt från ett vallprov (från första studien) och två foderprover (från andra studien) och därefter kördes PCR på provet för att amplifiera de mögelsekvenser som eventuellt fanns. Dock kan inte alla mögelarter (exempelvis *Aspergillus*, *Fusarium* och *Penicillium*) artbestämmas i ITS-regionen och därav är andra regioner också utav intresse. Nya sekvenseringsmetoder finns nu tillgängliga och 454-sekvensering används inte längre men de primers som testades i denna studie används i de nya sekvenseringsmetoderna.

Popular science summary

Mould growth in wrapped bales in Norway and Sweden

Today, haylage is a common forage for ruminants and horses in Norway and Sweden. Haylage is often referred to as wrapped forage with dry matter (DM) content between 50 and 84 %, and is preserved through a combination of drying and airtight storage. It differs from the wetter silage in several aspects, as the higher DM content results in restricted lactic acid bacteria fermentation. This means that the concentration of fermentation products will be lower and pH will be higher in haylage compared to silage. Haylage is also less compacted and more porous than silage, and therefore air-tight storage with intact wrapping is important for the preservation and to avoid fungal growth in the forage.

If oxygen enters the bale, for example by leaking through untight polyethylene film layers, filamentous fungi (mould) starts to grow. As some moulds can produce harmful spores and/or toxins (mycotoxins), it is important to avoid their growth in feed. Therefore a study has been performed where the aim was to identify mould species in wrapped forages, and to investigate whether different factors in production and management of bales (including nutritional content of the feed) were associated with increased risk of presence of mould and mycotoxins in haylage.

In the first study, the microbial load on the crop before harvest and in the haylage after conservation was compared. This comparison was repeated at three harvest dates (June, July, August) which were all comprising the primary growth. The amount of mould in the herbage were higher in July compared to June, but not compared to August. The amount of mould in the haylage samples were highest in haylage from August and lowest in June. There were no differences between June and July, or July and August.

The mould species detected in the herbage samples were not the same as the species detected in the haylage, and number of moulds was lower in haylage

compared to herbage. This may indicate that during the preservation of haylage, a selection of some mould species occurs. The results also showed that a late harvest date may increase the mould load on the herbage, but it is not necessarily transferred to the haylage.

In the second study samples from wrapped forages with DM content were collected during two years from 124 farms, 25 from Norway and 99 from Sweden. On each farm, samples were taken from three bales by use of a core sampler. The polyethylene film layers were removed from one of the three bales and if there was any visible mould patches on the bale surface, it was recorded and sampled. At the same time as sampling of the bales, information about production and storage of the bales was obtained from the producer. Mould occurrence was analysed with three different methods: Method I, samples of visible mould patches; Method II, small pieces of plant material from core samples were placed on agar plates for detection of mould species (direct plating); and Method III, dilution series was made from core samples, which were inoculated on agar plates for determination of the amount of moulds in the wrapped forage. Mould species were identified by cultivation on selective substrates and microscopic characteristics, as well as by DNA sequencing. Mycotoxin concentration was analysed using core samples obtained with method III.

The results showed that more than half (52 %) of the farms had visible mould patches on the bale surface (Method I). A total of 52 fungal species were detected. The most common species in Method I was *Penicillium roqueforti*, detected on 20 % of the farms. Other species found on the bale surface was *Arthrinium* spp (18 %), *Aspergillus fumigatus* (6 %) and *Fusarium poae* (6 %). In Method II, where direct plating was performed, mould was detected on 79 % of the farms. The most common species/genera was *Arthrinium* spp., (47 %), followed by *P. roqueforti* (28 %) and *Sordaria fimicola* (25 %). In Method III, where dilution plating was performed, mould was detected on 56 % of the farms. The most common species/genera *P. roqueforti* (28 %) followed by *Arthrinium* spp. (15 %), *A. fumigatus* (7 %) and *Eurotium herbariorum* (7%).

Eleven mycotoxins were analysed and nine of these were detected in 39 % of the samples. The three most common mycotoxins were enniatin-B (14 % of the samples), deoxyivalenol (12 % of the samples) and beauvercin (10 % of the samples). It was common to find more than one mycotoxin in the same sample.

The present study showed that mould occurrence was common in bales sampled on the farms. Increased risk of mould presence in wrapped forages was explained by several management and production factors. Earlier harvest date and wide-spreading of the fresh herbage during wilting led to reduced risk of mould presence compared to later harvest date and wilting the crop in windrows.

Also, low tightness of the bales was a factor that increased the risk of visible mould patches on the bale surface.

Using fewer than eight layers of stretch film increased the risk of mould occurrence, while more than eight layers decreased the risk of occurrence The probability of finding mould was over 0.65 in forage with DM contents between 60 to 80 % DM even if more than eight layers of polyethylene stretch film was used.

In the third study, three new primers for the fungal gene ITS (internal transcribed spacer) were tested for 454-sequencing, a pyrosequencing method. With this method, DNA was extracted directly from the forage sample and thereafter PCR was performed to amplify the fungal sequences that might be present. However, not all fungi (such as species belonging to genera *Aspergillus*, *Fusarium* and *Penicillium*) can be identified to species level in the ITS-region, and therefore other fungal regions and other sequencing methods are of interest. However, the primers tested in the current study are now in use with new sequencing methods.

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