CHARACTERIZATION OF FORAGE MOLDING DURING STORAGE AND ITS EFFECTS ON PREFERENCE, RUMEN ENVIRONMENT AND DEGRADABILITY OF FEEDSTUFFS

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by

Michael Undi

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CHARACTERIZATION OF FORAGE MOLDING DURING STORAGE AND ITS EFFECTS ON PREFERENCE, RUMEN ENVIRONMENT AND DEGRADABILITY OF FEEDSTUFFS

BY

MICHAEL UNDI

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Undi, Michael. Ph.D. The University of Manitoba, February, 1995. Characterization of forage molding during storage and its effects on preference, rumen environment and degradability of feedstuffs. Major **Professor:** K. M. Wittenberg.

Studies were conducted to evaluate changes in forage constituents and fungal species in alfalfa forage baled and stored at 64.1-66.2% (low), 71.9-73.2% (medium) and 75.4-77.4% (high) DM contents. The role of fungal biomass in moldy alfalfa hay on preference and rumen function also was examined. Most changes in forage constituents occurred within two weeks of storage and were related to initial DM content at baling and temperature during storage. Rate of increase of DM, NDF, ADF, ADIN and glucosamine was highest ($P<0.05$) in low DM forage. Peak temperature reached was highest ($P<0.05$) in low DM forage and lowest ($P<0.05$) in high DM forage. Moisture content at baling did not influence ($P>0.05$) total fungal counts, number of species, species diversity or dominance in the early phase of storage (days 1 to 8). In the later phase (days 9 to 60), total fungal counts were highest ($P<0.05$) in low DM forage, and number of species highest in medium DM forage. Species dominance was highest in high DM forage. Fungal succession, in which field fungi were replaced by storage fungi during storage was shown. Common fungal species in the early storage phase were *Phoma*, *Cladosporium*, *Alternaria* and yeasts. In the later phase, *Aspergillus repens*, *Absidia* spp. and some yeasts were associated with medium and high DM forages while *Emericella nidulans*, *Aspergillus fumigatus*, *Absidia* spp. and thermotolerant hyphomycetes were more predominant in low DM forage. The correlation between visual mold and glucosamine, an estimate of fungal biomass, was low (r=0.40). Fungal biomass in hay harvested and stored under diverse conditions can be estimated by NIRS. In calves, preference of hay declined as either the fibre content ($P<0.05$) or the amount of fungal biomass in hay increased ($P<0.05$). Increasing fungal biomass content in alfalfa hays with similar nutrient profiles did not influence ($P>0.05$) rate and extent of DM degradation in corn grain, barley grain, canola meal or alfalfa hay. Rate and extent of CP degradation in canola meal and alfalfa hay, and rate and extent of NDF degradation in alfalfa hay and barley straw also were not influenced ($P>0.05$) by fungal biomass in alfalfa hay. Current methodology used to determine the feed value of alfalfa hay that has undergone molding can be improved.

FOREWORD

Preparation of this thesis is in manuscript format. Manuscripts I and II will be submitted to Can. J. Plant Sci.. Manuscript III will be submitted to the Can. J. Anim. Sci.. Manuscript IV will be submitted to J. Dairy Sci. while manuscript V will be submitted to Anim. Fd. Sci. Tech. The authors of manuscripts I, IV and V are Undi, M. and Wittenberg, K. M.. The authors of manuscript II are Undi, M., Wittenberg, K. M. and Holliday, N. J. and for manuscript III the authors are Undi, M., Wittenberg, K. M. and Williams, P. C.

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GENERAL INTRODUCTION

Approximately 5.8 million hectares were used to produce about 29 million metric tonnes of tame hay in Canada in 1991 (Statistics Canada 1992). Fifty-six percent of the land was used to produce 16.4 million metric tonnes of alfalfa (*Medicago sativa* L.) and alfalfa-grass hay, with an estimated value of $1 billion. Only a small fraction (0.2%) of total hay produced was exported, the rest presumably being used to feed livestock on the farm or sold within the country. It is impossible to estimate how much of the exported hay was alfalfa since export figures are not broken down into forage species. One determinant of hay price, be it for export or local trade, is hay quality, and quality valuation takes into account nutrient composition as well as visual assessment for color and mold.

A major objective in hay-making is to conserve the yield and nutritional value of fresh-cut forage by drying it as quickly as possible to a level at which the activity of microbial decomposers is halted (Macdonald and Clark 1987). Forages, as hay or silage, are a major component of ruminant diets and can contribute more than half of the animals' dietary protein requirements (Kennelly and Baars 1990). Forage conservation is important for providing feed for ruminants and this becomes magnified in situations where animals do not have access to grazing. Examples would be during winter months, during periods of drought or in zero grazing systems. Excess forage during periods of rapid growth also can be harvested and stored while in good quality, thereby reducing wastage from trampling by grazing animals.

Conservation of forage as hay is risky because of dependence on good hay-making weather. Forage has to be dried to a DM content of approximately 80-85%, which normally requires 2 to 10 days of good drying weather without rain (Jones and Harris 1980; Lingvall and Nilsson 1980; Robertson 1983; Fonnesbeck et al. 1986). Excessive forage drying can lead to DM losses in the field, and leaves are the most susceptible part of the plant to be lost during prolonged drying. There is, therefore, a need to investigate methods which can be used to reduce exposure of wilting forage to the environment while ensuring high yields and good quality hay. Under poor drying conditions forage may be stored when DM content is low. Such storage, however, results in growth and proliferation of storage fungi. Common species of storage fungi include mainly *Aspergillus* , *Mucor* and *Absidia* (Gregory et al. 1963; Breton and Zwaenepoel 1991). Storage fungi reduce forage quality by utilizing nutrients in forage as an energy source and also their metabolic activity leads to evolution of heat which can reduce availability of protein in stored forage (Cherney et al. 1987; Collins et al. 1987).

Hay is consumed by animals and used mainly as a source of energy. Therefore, quality must be determined by those characteristics that affect consumption and utilization by animals. Important characteristics that determine hay quality include, stage of maturity, forage species, chemical composition, leaf to stem ratio, physical form, damage or deterioration during harvest and storage, and the presence of antiquality constituents such as alkaloids and other toxic constituents (Lechtenberg and Hemken 1985). Bohstedt (1951) defined good quality hay as being leafy, having a bright green color, a pleasant odor or aroma, high nutrient value and palatability. Fungal biomass in hay maybe an important non-nutritive factor but this aspect is rarely discussed when problems of nutrient utilization and intake in stored forages are considered. Fungal biomass in hay increases amount of dust (spores and mycelia) and also leads to a discoloration of hay, which is associated with overheating during storage.

There has been very little work on the effect that fungal biomass *per se* has on animal performance, most work in this area having concentrated on DM and nutrient losses during storage. Available work indicates that there are no differences in intake when animals are fed moldy versus nonmoldy hay and nutrient digestibilities are lower on moldy hay. A limitation in available work on moldy hay is the way different researchers have defined degree of moldiness. Visual assessment of moldiness is the prevalent method. As a result, comparisons across experimental results are difficult. In this work a relatively more quantitative method which assays glucosamine, a fungal cell wall component, was used to estimate amount of fungal biomass in hay.

The overall objective of the work undertaken was to characterize the effect of fungal biomass on post-storage hay quality. Specific objectives were:

1) To determine changes in forage constituents and to characterize development of fungi in alfalfa forage baled and stored at different DM contents.

2) To verify use of near infrared reflectance spectroscopy to estimate amount of fungal biomass in hay under diverse harvest and storage conditions.

3) To determine whether fungal biomass in molded alfalfa hay affects preference, and hence, palatability, when the hay was fed to calves with no previous exposure to molded feedstuffs.

4) To establish whether fungal biomass typically observed in molded alfalfa hay would affect digestion kinetics of feedstuffs in the rumen.

REVIEW OF LITERATURE

Forage drying in the field

Forage is cut and left in the field for a period of time to allow wilting until such time as it reaches a desired DM. Successful storage requires wilting forage to a DM content of approximately 80-85%, and forage dries to this DM content in 2 to 10 days with good drying weather and limited exposure to rain (Jones and Harris 1980; Lingvall and Nilsson 1980; Robertson 1983; Fonnesbeck et al. 1986). The period of wilting is the critical stage in hay-making since it determines the amount of time forage is exposed to the environment, with all the risks associated with that exposure.

Rate of water loss during wilting follows an exponential decay pattern so that the rate progressively decreases as DM content of the material increases. Therefore, the amount of time necessary to increase DM content from 20% to 30% is considerably less than the time required to increase DM content from 70% to 80% (Wilkinson 1981). About 75% of the moisture in wilting forage is lost in the first fifth of the drying time (Jones and Harris 1980). Rapid initial drying occurs when the stomata are open, plant resistance is minimal, and the vapor pressure deficit between plant tissues and ambient air is maximal. During the last phase of drying, when DM is considerably higher (>45%), water in the plant is bound by osmotic and matrix forces and is, therefore, difficult to dislodge (Macdonald and Clark 1987).

Plant and environmental factors that affect forage drying

Field drying of forage depends on environmental conditions, but the drying behavior of plant material is affected by its species, stage of growth, leaf to stem ratio, and structure of the swath which acts as a barrier to the removal of the water lost from plant tissue (Klinner and Shepperson 1975). Within the swath, a microclimate develops, which imposes a limitation on water loss. Drying is more rapid on the surface of the swath and, due to poor air circulation through the swath, rate of water loss is lower in the middle of the swath. Relative humidity values within the swath during the earlier stages of wilting are rarely below 80% and humidity inside a swath depends on ambient humidity, water content of the crop and airflow through the swath (Jones and Harris 1980).

Rate of water loss is greater in leaves than in stems in both grasses and legumes. Leaves dry at a more rapid rate than stems by a factor of 10 to 15 times, and 30% of the water in stems is lost through the leaves (Murdoch 1980). Initial moisture content of forage varies with forage species, crop maturity, weather conditions prior to cutting, and with time during the season (Wilkins 1988). Due to a higher initial moisture content and a larger proportion of stem to leaf dry weight, legumes tend to dry more slowly than grasses (Jones and Harris 1980). But even within legumes, alfalfa dries relatively quickly, and is thus the species of choice for hay-making, while the much slower drying of red clover (*Trifolium pratense* L.) encourages its conservation as silage (Macdonald and Clarke 1987). Grass species, at a similar growth stage and water content, have been shown to vary in the rate at which they lose water after cutting (Jones and Harris 1980). For example, tall fescue (*Festuca arundinacea* L.) is notable for its rapid rate of water loss, drying up to four times faster than perennial ryegrass (*Lolium perenne* L.).

In terms of environmental conditions, solar radiation, temperature, wind speed, relative humidity, and soil moisture content are the most important factors that determine rate of drying (Savoie and Mailhot 1986). Solar radiation has the greatest influence on water loss in both legumes and grasses, although only part of the incoming radiation is absorbed by the crop (Savoie and Mailhot 1986). Wind removes water vapour from boundary layers and so accelerates transpiration (Klinner and Shepperson 1975). Relative humidity creates the gradient which influences water loss from drying forage and, because hay is hygroscopic, relative humidity also influences the equilibrium moisture content of forage approaching a safe storage moisture content. The relative humidity which creates the gradient that influences water loss is that within the swath adjacent to the plant tissue (Macdonald and Clark 1987). Soil moisture content also influences crop drying rate and the vertical moisture gradient within the herbage layer (Klinner and Shepperson 1975).

Improving rate of forage wilting

Water loss in wilting forage can be facilitated by a number of methods which have been discussed in detail by Macdonald and Clark (1987). Methods include growing grasses and legumes in mixtures to lessen the impact of slower drying legumes. Water loss also can be improved by leaving a tall stubble to lessen soil contact and promote air movement under and within the swath. Cutting small fields on each of several days lessens the risk of rainfall damage. Swath treatments such as tedding and turning improve rate of water loss (Nash 1985).

Drying rate may also be greatly accelerated by crop conditioning. Crop conditioning was defined by Klinner and Shepperson (1975) as the practice of damaging the plant's surface to release moisture readily to increase evaporation rate of crop moisture. There are two types of conditioning, mechanical and chemical conditioning. Mechanical conditioning involves crushing and crimping by pressure rollers, and laceration by flails. Crushing causes longitudinal splitting and localized bruising. Crimping produces intermittent splits and bruises while flail treatment has a random effect and is the most severe (Klinner and Shepperson 1975).

Chemical conditioning involves use of chemicals such as potassium carbonate and potassium hydroxide (Hong et al. 1988). The chemical agents alter cuticular resistance and thus enhance drying rate. Faster drying improves both yield and quality of hay by reducing duration of respiratory losses, lessening the risk of weather damage, and hastening forage removal from the field (McGechan 1988).

Losses during hay-making

Losses during hay-making can be divided into two, field and storage losses, and generally an effort to reduce one will result in an increase in the other. For example, baling and storing forage at 60% DM can reduce field DM losses by 50% or more but greatly increases storage losses (Nash 1985; Wilkins 1988). On average, about 20% of original DM is lost in the field. Where weather conditions are such that forage can be dried rapidly and sufficiently for storage, DM losses can be as low as 10% (Nash 1985). Total DM losses under wet and humid climatic conditions can exceed 30% of the initial DM (Rees 1982).

Dry matter losses in hay-making are larger than those in other important crops and would be considered excessive and unacceptable in such crops (Koegel et al. 1985). In practice, farmers pay little attention to DM losses in forages. Since most forage is used on the farm rather than marketed, the monetary value of these losses is not fully appreciated. Many problems associated with hay-making could be solved if forage could be dried quickly in the field or if it could be stored at low DM without spoilage (Lechtenberg and Hemken 1985)..

Field losses

Field DM losses range from 1 to 3% per day for crops harvested for silage, but can reach 4% per day for forage dried under poor weather conditions to 80% DM content (Wilkinson 1981). These field losses can be reduced by minimizing the period of time between cutting the forage and removing it from the field. Considerable loss of leaf material can occur when forage is dried to the extent that its DM content is above 70% and this is more critical under unfavourable conditions (Alli et al. 1985). Field DM losses occur during wilting as a result of plant respiration and microorganism activity, leaching by rain, and leaf shatter due to mechanical treatment of the crop (Jones and Harris 1980; Lingvall and Nilsson 1980). The magnitude of loss from each of these causes increases as drying is prolonged, and efficient hay-making depends largely on the speed with which water is removed to a level which inhibits the activity of enzymes of the plant and microorganisms (Jones and Harris 1980).

Loss due to respiration

After cutting, the forage crop continues to respire but at a declining rate until it is inhibited by either low moisture conditions or by lack of respiratory substrate in case of extended respiratory activity due to wilting under poor drying conditions (McGechan 1989). Water-soluble carbohydrates are the principal group of compounds that are utilized in both plant and microbial respiration (Rees 1982; McGechan 1989). Alli et al. (1985) reported a 7% decline in water-soluble carbohydrate content in timothy after 21.5 hours of drying. The decline in water-soluble carbohydrate content was attributed to a decline in photosynthetic activity and a simultaneous increase in plant and microbial respiratory activity. Pizarro and James (1972) estimated that approximately 20% of water-soluble carbohydrate in cut grass was lost due to respiration.

Several factors affect rate of respiration of wilting forage. Respiration rate declines as DM content increases (Wilkinson and Hall 1966; Wood and Parker 1971; Pizarro and James 1972). Wilkinson and Hall (1966) reported a zero respiration rate when DM content of alfalfa forage was approximately 90%. At such high DM contents, plant enzymatic activity ceases or at least drops below a detectable level.

Rate of respiration in alfalfa forage increases with an increase in temperature, reaching a maximum at 26.7°C (Wilkinson and Hall 1966). As the temperature continues to increase, respiration rate will reach this maximum and then begin to decrease due to coagulation of protein, death of cells, and destruction of contributing microorganisms. Wilkinson and Hall (1966) found respiration rate to be zero at 3.9°C.

Effects of plant maturity on respiration rate were reported by Pizarro and James (1972). Stage of growth had a marked influence on respiration rate, the younger the plant material the greater the respiration rate. In terms of forage species, Wilkinson and Hall (1966) reported a tendency for higher respiration rate in wheatgrass compared to alfalfa forage, at an equivalent stage of growth. During wilting, forage is subject to rain damage. Pizarro and James(1972) showed that re-wetting forage can prolong respiration and hence increase losses.

Respiration rates determined in wilting forage represent the sum of respiration of plant tissues and that of microbial flora associated with the surface of plant materials (Pizarro and James 1972). The source of soluble carbohydrate for the respiration of the microbial flora must be the plant material so that one is still dealing ultimately with loss from forage material.

Losses due to rainfall

In addition to prolonging the wilting period, rainfall also results in direct loss of soluble components through leaching (Collins 1983; Fonnesbeck et al. 1986). Leaching removes the most soluble materials, notably available carbohydrate, soluble N, soluble minerals e.g. potassium, and lipids (Fonnesbeck et al. 1986). Exposure of dry forage to rain can also result in leaf loss by leaf shatter, an effect which is more critical in legumes than grasses (Alli et al. 1985). The major factors which affect DM loss are the characteristics of the rain, including amount, intensity, and duration. Timing of the rainfall is perhaps more critical than the amount, as tolerance to rainfall declines with drying time, since relative integrity of the cuticle and cellular membranes in fresh cut forage prevents loss of soluble nutrients (Nash 1985; McGechan 1989). Crop factors such as DM content at the time of rain, maturity, leaf-to-stem ratio, swath density, and species are important in determining amount of damage due to rain (Rotz and Muck 1994).

Studies on effects of rain (wetting) on DM yield and quality of alfalfa and red clover forages showed that leaf loss was increased significantly by wetting and was 2 to 3 times that of forage of the same species without wetting (Collins 1983). Unwetted forage,

forage wetted during drying and forage wetted after reaching 85% DM lost 10.5, 43.4, and 53% of the initial DM, respectively. Total nonstructural carbohydrate in both species declined by 71% in wetted forage.

Sprinkling alfalfa forage with up to 20 mm of simulated rain (sprinklers) increased the plant cell wall constituents from 39 to 44%, by leaching out soluble constituents but did not change CP content of the forage (Fonnesbeck et al. 1986). Nonstructural carbohydrate was most vulnerable and accounted for 70% of the loss of cell contents. Hay quality was reduced more by rain than by advancement in forage maturity.

Rain during forage wilting can lead to increased build-up of fungal biomass in the field. Exposure of alfalfa forage to 34.1 mm of rain while still in the swath resulted in an increase in fungal biomass of more than 180% (Wittenberg et al. 1989). Increase in fungal biomass would in turn increase loss of soluble carbohydrate since the source of carbohydrate for fungal respiration is plant material (Pizarro and James 1972).

Mechanical losses

Another source of field DM losses is fragmentation caused by the action of machinery on plant tissues, mainly leaves, during cutting, swath treatment and harvesting (Wilkinson 1981). The magnitude of these losses depends on DM content of forage at baling, crop species, maturity, leaf-to-stem ratio and swath structure. As the crop dries, it becomes more brittle and its susceptibility to leaf loss rises. As a result, it becomes important to apply only gentle mechanical treatments during the later stages of field drying when DM content exceeds 50 to 60% (McGechan 1988; McGechan 1989). Leguminous crops such as alfalfa and red clover are subject to greater leaf loss than are grasses.

Even under the most favourable conditions, leaf loss of around 15% of the weight of total crop can occur in alfalfa. In grasses this may be as low as 2 to 5% of crop weight (Nash 1985).

Dry matter and nutrient losses in storage

Baling and storing forage at a low DM content reduces chances of rain damage by decreasing field drying time. Leaf retention is also increased thus reducing nutrient losses during drying (Fonnesbeck et al. 1986). However, such forage is likely to undergo molding with accompanying reduction in hay quality (Nehrir et al. 1978). Much of the DM that is lost during storage is non-structural carbohydrate and nitrogen compounds that are transformed into microbial biomass, carbon dioxide and water, and loss is proportional to initial DM content of forage entering storage (Kassperson et al. 1984; Rotz and Abrams 1988). Theoretically, the moisture content at which all microbial and biochemical action in stored forage is halted is 10-12% (Nash 1985) but such moisture contents would result in excessive leaf loss.

Alfalfa forage that was stored at three DM contents of 80-89%, 75-80%, and 66-75%, showed DM losses which were inversely related to initial DM content of forage entering storage (Rotz and Abrams 1988). Forage stored at 80-89% DM lost 4.5% DM over the storage period while at 66-75%, more than 10.9% DM was lost.

Dry matter loss in alfalfa forage stored at a low DM (74.1%) was approximately twice that of forage stored at a high DM (84.3%) (Cherney et al. 1987). Also, at the end of storage, total nonstructural carbohydrate content was lower and structural carbohydrate content higher in low DM forage.

Data compiled from different sources shows a relationship between DM losses in storage and DM content of alfalfa forage at baling (Fig. 1). At forage DM content of 80%, DM losses are around 5% and are higher for forage baled at lower DM contents (Fig. 1). Dry matter losses in grass forages were reviewed by McGechan (1990) and are lower than in legumes. With limited data, crude protein losses in storage are variable (Fig. 2).

Temperature changes during hay storage

The amount of heating that occurs during storage of baled forage is related to the initial moisture content; temperature increases almost linearly with increasing moisture content (Festenstein et al. 1965; Miller et al. 1967; Collins et al. 1987; Breton and Zwaenepoel 1991). Heating is also likely to be more in a stack of bales than in individual bales due to better ventillation of individual bales, as was demonstrated with grass forage (Gregory et al. 1963). During the early phase of storage, heat produced by continued plant respiration can be beneficial in that it can aid the removal of moisture, which might otherwise provide a medium for subsequent bacterial and mold growth (Wilkinson 1981).

Two characteristic temperature peaks have been described during forage storage. The first peak, which occurs within one to three days of baling, has been attributed to plant cellular respiration (Gregory et al. 1963; Breton and Zwaenepoel 1991).

Figure 1. Dry matter losses in alfalfa forage baled and stored at different DM contents.

Data from: Thorlacius and Robertson (1984); Atwal et al. (1986); Cherney et al. (1987); Rotz and Abrams (1988); Wittenberg et al. (1989); Wittenberg and Moshtaghi-Nia (1990); and Wittenberg (1994).

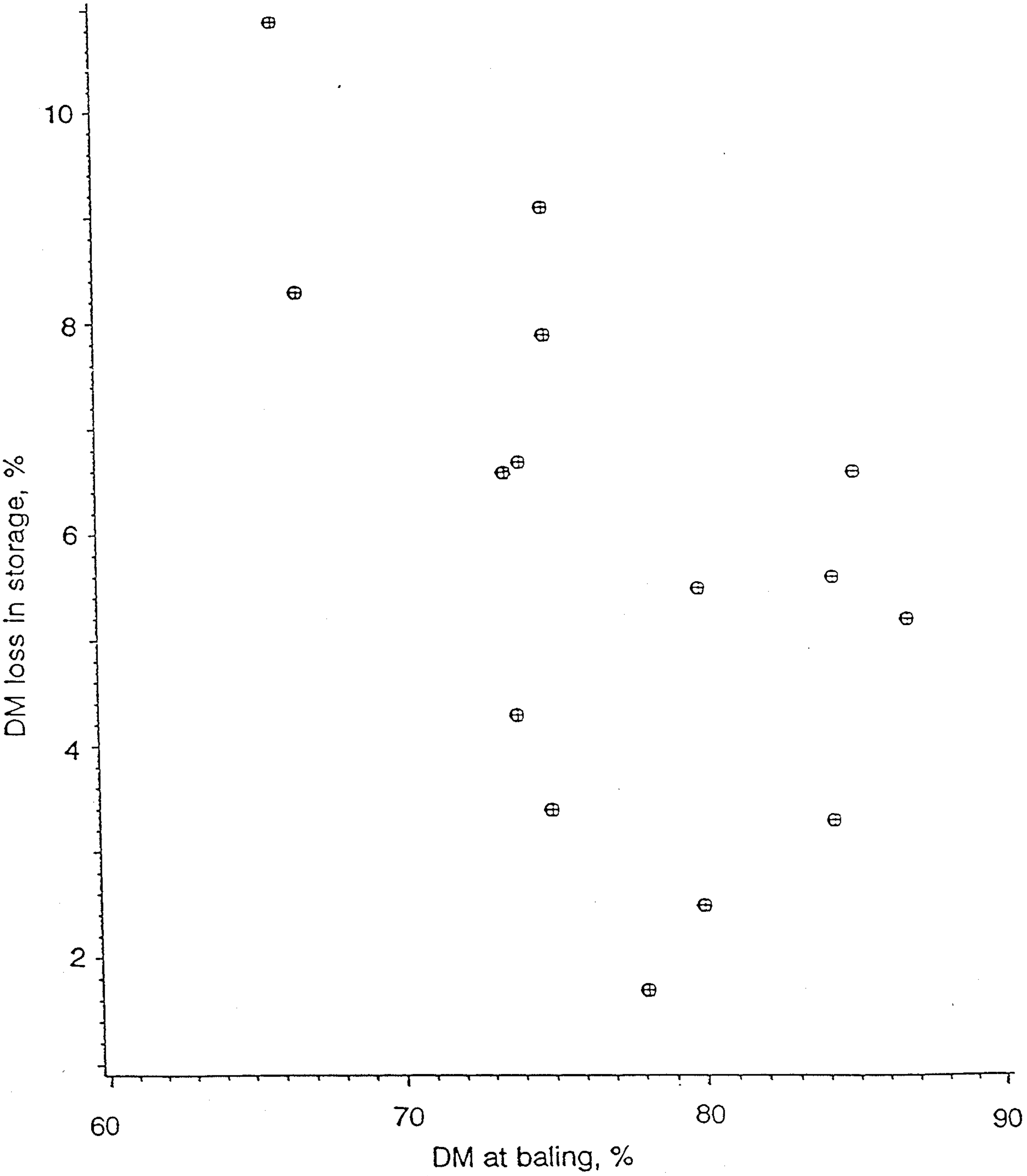
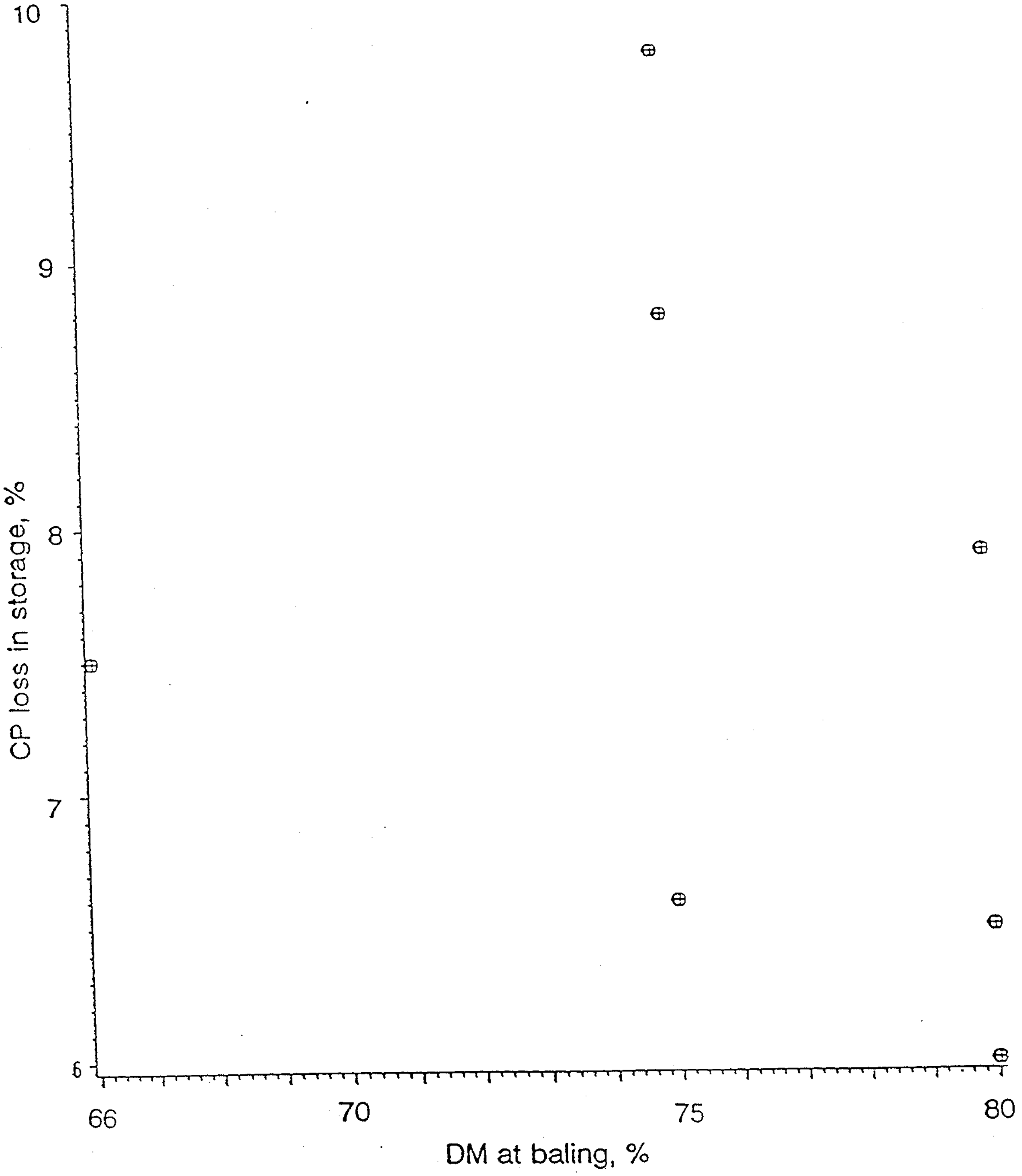

DM loss in storage, %
10
8
6
4
2
60
70
80
90
DM at baling, %

Figure 2. Losses of crude protein in alfalfa forage baled and stored at different DM contents.

Data from: Rotz and Abrams (1988); Wittenberg and Moshtaghi-Nia (1990); and Wittenberg (1994).


10
9
8
7
6
CP loss in storage, %
66
70
75
80
DM at baling, %

The second peak, which is higher and more persistent than the first, occurs much later and this second peak is due to respiration of rapidly developing fungi (Gregory et al. 1963; Breton and Zwaenepoel 1991). Temperatures of up to 65 and 70°C in stored forages are caused by fungal respiration. Above that, temperatures cannot be attributed solely to activity of microorganisms but to purely chemical reactions (Gregory et al. 1963).

Breton and Zwaenepoel (1991), comparing round bales of fescue forage which were baled at 54 and 73% DM, showed temperatures which were related to initial DM. Thermogenesis was higher for lower DM forage, reaching a peak temperature of about 65°C, and the thermogenesis consisted of two distinct phases: a very rapid and transient increase followed by a slower and more intense heating process lasting over 75 days.

Under laboratory conditions, Festenstein et al. (1965), demonstrated that increasing water content accentuated the heating pattern of forages stored in dewar flasks. Forage stored at 70% DM or more did not heat above 30°C, forages at 60% DM heated to 57-67°C while at 66-71% DM, forages heated to temperatures of between 33 and 55°C.

In alfalfa forage stored at 74.1 and 84.3% DM, peak temperature of dry-harvested forage was 31°C, while that of low DM forage exceeded 38°C in the initial stages (Cherney et al. 1987). Maximum temperature for low DM forage was reached after 7 days in storage and was 21°C higher than the temperature in high DM forage.

Microbial changes during storage

The standing crop, before it is cut and baled, carries a wide range of fungal species, which have been termed field fungi because they require free moisture for growth (Nash 1985; Lacey 1989). Field fungi (Table 1) are derived from soil by rain-

TABLE 1. Predominant fungal species in forage crops before harvest and during wilting.

Forage	Fungal spp.	Reference
Grass	*Cladosporium*, *Epicoccum*, *Alternaria*, *Acremoniella atra*, *Trichothecium*	Gregory et al. (1963).
Perennial ryegrass/red clover	*Fusarium*, *Cladosporium*, *Phoma*, *Alternaria*, *Mucor*, *Penicillium*	Pizarro and Warboys (1978).
Timothy/clover/meadow fescue	*Cladosporium*, *Fusarium*, *Alternaria*, *Botrytis*	Clevstrom et al. (1984).
Grass	*Fusarium*, *Cladosporium*, *Alternaria*, *Acremoniella*, *Botrytis*, *Trichoderma*	Kaspersson et al. (1984).
Fescue	*Fusarium*, *Cladosporium*, *Alternaria*, *Phoma*, *Colletotrichum*, *Ascochyta*, *Phaeoseptoria*	Breton and Zwaenepoel (1991).

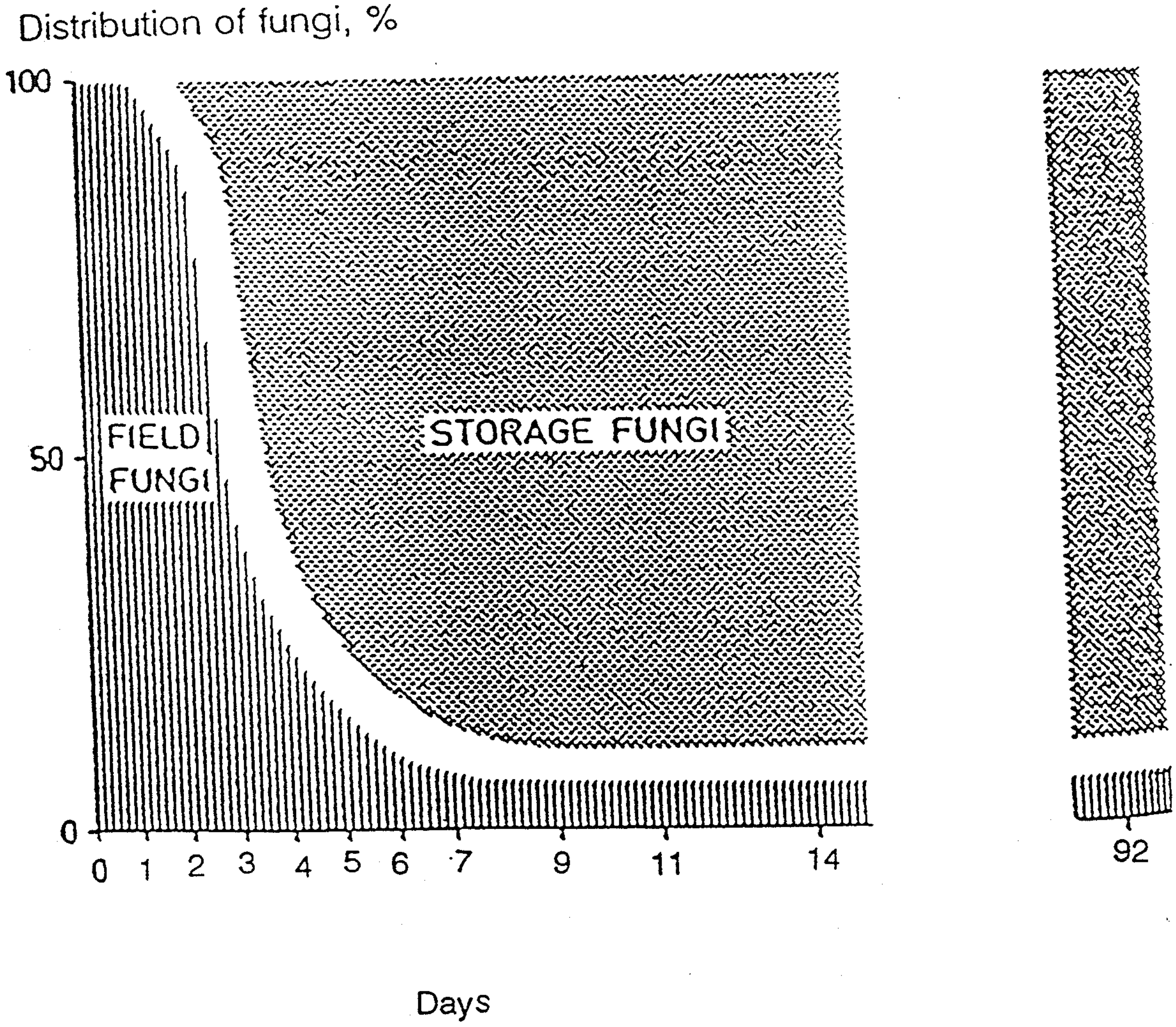
splash or deposited from air, or through epiphytic growth on living leaves or saprophytic growth on dead or senescent material (Gregory et al. 1963; Lacey 1975). Numbers of fungi on the standing crop will vary with environmental conditions. In alfalfa, Lin et al. (1992) reported temperature to be the major factor influencing epiphytic microflora. High average daily temperatures during a 28-day regrowth period prior to mowing significantly increased numbers of yeasts and molds. Yeasts and mold counts in the windrow also were related positively to temperature during wilting.

Fungal species which are considered to be storage fungi such as *Penicillium*, some members of the *A. glaucus* group (*A. amstelodami*) and *A. fumigatus* were isolated from forage left to wilt for 250 hours in the field, albeit in very low numbers (Pizarro and Warboys 1980). Also, under certain conditions, as in humid tropical areas, *A. flavus*, which is a storage fungi, will occur in significant numbers in the field (Lacey 1989).

During storage, field fungi are replaced by storage fungi, so called because of their ability to grow in low moisture environments. This transition occurs early in storage within the first week (Fig. 3; Nash 1985; Breton and Zwaenepoel 1991). Field fungi are mesophilic and are eliminated rapidly due to their thermosensitivity (Breton and Zwaenepoel 1991). Field fungi may sometimes persist in storage if forage is dry enough to prevent growth of typical storage fungi (Gregory et al. 1963; Lacey 1989).

Figure 3. Schematic graph showing the distribution of field and storage fungi during storage of untreated hay.

(Kaspersson et al. 1984).


Distribution of fungi, %
100
50
0
FIELD FUNGI
STORAGE FUNGI
0
1
2
3
4
5
6
7
9
11
14
92
Days

Both before harvest and during storage, growth of filamentous fungi is determined by the environment, especially by water availability, temperature, gaseous composition; and also by interactions with other microorganisms (Lacey 1989). In fescue forage baled at 54.8 and 72.8% DM, Breton and Zwaenepoel (1991) showed that most field fungal species were eliminated within thirty-six hours of storage. The major storage fungi that were isolated in low DM forage were thermotolerant species, which included *Absidia corymbifera*, *Aspergillus fumigatus*, *Humicola lanuginosa* and *Rhizopus* spp.

Grass forage that was baled at different DM contents showed clear differences in type of fungal species that developed during storage (Gregory et al. 1963). Forage stored at 54-60% DM content developed large numbers of *Mucor pusillus*, *Absidia* spp., *Aspergillus fumigatus* and *Humicola lanuginosa*. These species are thermotolerant and thrive at temperatures of 60-65°C. Forage stored at 65-70% DM content was molded mainly with *Mucor pusillus*, *Absidia* spp., and fungal species belonging to the *Aspergillus glaucus* group. At 75% DM, forage heated to 45°C and was molded mainly by mesophilic fungi of the *A. glaucus* group. Only in high DM forage (83-85%) was microflora sparse and more mixed in species composition.

In Sweden, *A. flavus* and *A. glaucus* were the predominant fungal species in grass forage stored at 69% DM (Kassperson et al. 1984). Bacteria species in the early stages were mostly gram-negative but as storage proceeded, gram-positive species became dominant, since this group can adapt to a wide range of water activity.

Festenstein et al. (1965), using an *in vitro* method showed fungal succession due to different DM contents in dewar flasks. At 74% DM, the dominant fungal species were members of the *A. glaucus* group, while at 69-72%, *A. nidulans*, *Absidia* spp., *A. versicolor*, and *Scopulariopsis brevicaulis* were predominant. At 60% DM content, *A. fumigatus* and *Humicola lanuginosa* were dominant.

Studies evaluating fungal species on the standing forage crop, the harvested crop during wilting and storage have mainly been undertaken under European conditions with grass forages. Very little such work has been done under North American conditions, where alfalfa is a common hay crop. In studies such as those cited above, DM contents at which most forages were stored were low, approaching DM contents aimed at when making haylage or even silage. Most forage intended for hay would be stored at about 80% DM for high DM hay and at about 70 to 75% DM for low DM hay.

Potential problems from feeding moldy hay

Diseases from moldy hay

Besides utilizing nutrients in forage during storage, fungal species in stored hay pose a risk to both individuals who handle moldy hay and livestock that are offered moldy hay (Lacey 1975; Nash 1985; Knudtson and Kirkbride 1992). The most serious threat of disease would seem to be from thermophilic fungal species and actinomycetes since body temperatures (37-39°C) are within the optimal range for growth of these organisms. Potential problems that can arise due to feeding moldy hay were reviewed by Lacey (1975) and Nash (1985). Spores of thermophilic actinomycetes such as *Micropolyspora faeni* and *Thermoactinomyces vulgaris* have been implicated in farmers' lung disease, a respiratory disease involving an allergic reaction (Nash 1985). In ruminants, diseases such as mycotic abortion, mycotic mastitis, and bovine lung disease have been traced back to moldy hay. *Aspergillus fumigatus* and *Absidia ramosa* also have been implicated in mycotic abortion and the same organisms and yeasts seem to be the cause of mycotic mastitis (Lacey 1975; Lacey and Dutkiewicz 1976; Knudtson and Kirkbride 1992). A nine year study by Knudtson and Kirkbride (1992) showed that *Aspergillus* spp. were the most frequently isolated fungal species in cases of mycotic abortion. *Aspergillus fumigatus*, in particular, accounted for most of the abortions.

Of concern, also, is the occurrence of allergies in humans due to handling moldy hay. Allergic reactions to moldy hay usually occur in the respiratory system with some occasional skin reactions (Lacey 1975). Field fungi such as *Cladosporium herbarum* and *Alternaria tenuis* are common causes of type I allergies.

Mycotoxin production in hay

Fungal species present in moldy hay produce mycotoxins under certain conditions, therefore the possibility of mycotoxins in moldy hay has to be addressed. Several factors interact to influence mycotoxin production and among these, moisture, temperature and pH are the most important (Council for Agriculture and Technology (CAST) 1989).

The presence of toxin-producing fungi itself is not a guarantee that mycotoxins will be produced, because few mycotoxins have been isolated from hay despite the presence of fungi capable of producing mycotoxins (Lacey 1975). A fungal estrogen (F-2) was isolated in moldy hay that was fed to dairy cattle and was implicated in causing infertility in a dairy herd (Mirocha et al. 1968). Production of aflatoxin B_1 and G_1 by *A. flavus* in haylage (55% DM) that was treated with formic acid was reported by Clevstrom et al. (1981). In this study, *A. flavus* occurred as a pure culture, which is not a common situation in hay. Lacey (1991) pointed out that *A. flavus* is seldom an important fungal species in hay.

Mycotoxins produced by storage fungi in conserved forages were reviewed recently by Lacey (1991). The group of storage fungi commonly found in hay are *A. glaucus* group (eg. *A. amstelodami*, *A. repens*, *Eurotium* spp.). These require little water for growth and occur in forage stored at up to 75% DM. This group has been discounted as being of little practical significance in terms of mycotoxin production (Lacey 1991) though they do produce some mycotoxins (Table 2). *Aspergillus* spp. in other agricultural products produce a diverse number of mycotoxins including aflatoxins, ochratoxins, and sterigmatocystin (CAST 1989). For example, *A. versicolor*, in addition to producing sterigmatocystin, can produce cyclopiazonic acid (CAST 1989; Lacey 1991; Table 2). *Fusarium* spp. produce zearalenone, and trichothecene mycotoxins that include: T-2 toxin, diacetoxyscirpenol, nivalenol, deoxynivalenol and fusarenon (CAST 1989; Lacey 1991). *Fusarium* spp. can colonize hay drying slowly in the field in wet weather but do not compete well with storage fungi under the conditions that usually prevail (Lacey 1991).

Effect of mycotoxins on ruminant animals

Ruminant animals, by the nature of their digestive system, offer some resistance to ingested mycotoxins. The complex microbial ecology of the upper gastrointestinal tract in ruminant species is the most frequently cited factor involved in the difference

TABLE 2. Common fungal species in hay and the mycotoxins they produce in other agricultural feedstuffs.

Fungal species	Toxin(s) produced
Alternaria spp.	alternariol, altenuene, tenuazonic acid
Fusarium spp.	zearalenone, T-2 toxin, diacetoxyscirpenol, nivalenol, deoxynivalenol
A. fumigatus	clavine alkaloids: fumigaclavine A and C, agroclavine, erymoclavine, festuclavine, tremorgens: verruculogen, fumitremorgens
A. versicolor	sterigmatocystin, cyclopiazonic acid, griseofulvin, nidulotoxin
A. nidulans	sterigmatocystin
A. flavus	aflatoxins

(CAST 1989; Lacey 1991)

between the toxicology of ruminants and that of monogastric species (Raisbeck et al. 1991). Research by different workers has shown the ability of rumen microorganisms to detoxify mycotoxins either *in vitro* or *in vivo.*

Trenholm et al. (1984) showed that feeding deoxynivalenol (DON)-contaminated wheat to dairy cattle resulted in slight decreases in feed consumption that returned to normal after a few weeks.

Using *in vitro* ruminal preparations, Westlake et al. (1989) showed that bacterial and protozoal preparations were capable of degrading toxins. T-2 toxin, HT-2 toxins and DON, were all degraded when they were added at a level of 10ug/ml. Kiessling et al. (1984) reported the metabolism of aflatoxin B_1, ochratoxin A, zearalenone, T-2 toxin, diacetoxyscirpernol (DAS) and DON by rumen microorganisms *in vitro.* Ochratoxin A, DAS, T-2 and zearalenone were degraded by rumen microorganisms, and protozoa were invariably more active than bacteria. Aflatoxin B_1 and DON were not degraded to a large extent, and only minor differences were observed between rumen fluid from sheep and cattle on rate of mycotoxin metabolism. In similar work, *in vitro* incubations of T-2, DAS and DON for periods of 12 to 48 hours showed that all three mycotoxins were biotransformed (Swanson et al. 1987).

Digesta from hay-fed sheep was shown to hydrolyze ochratoxin A *in vitro* up to 5 times faster than digesta from grain-fed sheep. *In vivo* degradation of ochratoxin A was more rapid in sheep fed a hay diet than a grain diet (Xiao et al.1991). These differences were attributed to types and numbers of rumen microorganisms which occurred on each diet.

There is no question that presence of mycotoxins in hay or conditions that might lead to their production in hay are possibilities which warrant further investigation.

Estimating amount of fungal biomass in hay

Assessment of health hazards from moldy hay requires reliable methods for estimation of amount of fungal biomass in hay (Lacey and Dutkiewicz 1976). Reliable methods of estimation also are required to assess animal performance, where animals have access to moldy hay. Important early research concerning feeding of moldy feedstuffs involved visual assessment of feed, and categorized feed as either moldy or nonmoldy (Jones et al. 1955; Mohanty et al. 1969). While such descriptions of feed treatments are adequate for individual experiments, the work cannot be repeated elsewhere or the following year by the same workers. Such descriptions have, over the years, been replaced by more quantitative methods. Even though recent methods are quantitative, they still provide only estimates of amounts of fungal biomass.

Relative mold index (RMI)

The relative mold index is a visual scoring method which attempts to go a step further by assigning scores to different bales of hay. A rating system is assigned to hays which differ in fungal biomass accumulation during storage. The RMI criteria can be used as follows: Values range from 1 to 5, with 1=no visible mold, 2=presence of spores between flakes, 3=presence of spores throughout the bale, 4=mycelial mat between flakes, and 5=mycelial mat throughout the bale (Roberts et al. 1987a). Many variations to assigning RMI values are used by different researchers. Reliable scoring results can be obtained if the procedure is carried out by two or more experienced technicians and the method gives good results for hay that is either extremely moldy or extremely clean (Roberts et al 1987a). Under current hay marketing procedures hay is sold on the basis of nutrient content and a visual assessment for mold, color, and dust.

Plate and spore counts

Fungal biomass in hay also can be assessed using traditional microbiological methods such as plate and spore counts (Gregory et al. 1963; Lacey and Dutkiewicz 1976; Cherney et al. 1987). Plate and spore counts have the advantage that they give specific numbers of the different species found in hay. Such methods, however, measure culturable fungi only and often require long incubation periods on selective agar media and as such, are not suitable for routine or rapid use (Tothill et al. 1992).

Ergosterol as an estimate of fungal biomass

Ergosterol (ergosta-5-7,22-trienol) is the predominant sterol in fungal cell membranes and is specific to fungi, being either absent or a minor constituent in higher plants and animals (Seitz et al. 1979; Naewbanij et al. 1984; Tothill et al. 1992). This has been taken advantage of in developing a method for estimating fungal biomass (Seitz et al. 1979). Seitz et al. (1979) found that the ergosterol assay was more sensitive to early growth and it could detect fungal growth before any visible mold could be detected.

In wheat grain, Tothill et al (1992) found a positive correlation between ergosterol content and total fungal biomass. Ergosterol content in grain increased prior to visible mold spoilage and the amounts of ergosterol in field and storage fungi differed considerably.

The ergosterol assay so far has been confined mainly to estimation of fungal biomass in grain though, Seitz et al. (1979), suggested that it can easily be adapted for use in other natural materials.

Glucosamine as an estimate of fungal biomass

Fungal biomass in various agricultural products has been estimated by determining the chitin content. Since chitin is a constituent of the cell walls of most fungi, it can be used as an estimate of fungal biomass in agricultural products (Donald and Mirocha 1977; Roberts et al. 1987a). Chitin, a polymer of N-acetyl-D-glucosamine, is commonly found in the exoskeleton of insects as well as in spores and mycelium of fungi (Donald and Mirocha 1977; Bishop et al. 1982). Chitin is measured as its hydrolysis product, glucosamine (Ride and Drysdale 1971; Donald and Mirocha 1977). Insects would likely raise the amount of chitin in the sample but this interference seems minimal. After contaminating tomato paste with insect fragments at levels 20 times above the accepted Food and Drug Adminstration (FDA) guidelines, Bishop et al. (1982) were able to show that glucosamine levels were raised only slightly, and they concluded that the presence of insect components in processed foods would not interfere with detection of mold, unless fungal concentrations were small.

The glucosamine assay has been used to estimate fungal biomass in infected barley and rye (Rotter et al. 1989), wheat grain (Nandi 1978), corn and soybean seeds (Donald and Mirocha 1977), and hay (Jones et al. 1985; Roberts et al. 1987a; Wittenberg et al. 1989). Processed products such as tomato paste also have been assessed for fungal biomass using this method (Bishop et al. 1982; Cousin et al. 1984). Variations in analysis by different researchers have been on type of hydrolysis employed, whether alkaline (Ride and Drysdale 1972; Roberts et al. 1987a) or acid hydrolysis (Rotter et al. 1989; Wittenberg et al. 1989). Plassard et al. (1982) reported that acid hydrolysis gave high yields, averaging 90%, whereas alkaline hydrolysis produced only 50% of glucosamine residues. Alkaline hydrolysis also required numerous washings to remove excess base.

Advantages of the glucosamine assay are that the assay will reflect total mycelium (viable and non-viable). The method is also relatively more rapid compared to methods such as plate and spore counts (Donald and Mirocha 1977; Bishop et al 1982). The disadvantage is that it does not identify the fungi involved. Sharma et al. (1977) found evidence to suggest that the chitin assay as applied to estimation of fungal biomass in aero-aquatic fungal species was of limited value since, in their work, chitin content was not related to fungal biomass, and varied with age, morphology and cultural conditions. Whipps and Lewis (1980) argued that this method can be used validly in comparative studies where only an indication of changes in fungal growth, not absolute fungal biomass content is required. Rotter et al. (1989) further argued that the method used by Sharma et al. (1977) was based on a colorimetric procedure which did not adequately separate glucosamine and galactosamine entities. Despite this, there is enough evidence to suggest that this assay gives useful estimation of fungal biomass in agricultural products.

Near infrared reflectance spectroscopy (NIRS)

A further step in the glucosamine assay was its adaptation for use with near infrared technology in assessing fungal biomass in hay (Roberts et al. 1987b) and in grain (Roberts et al. 1991). The use of NIRS in forage quality analysis has become almost routine and has been used for determining nutrients such as NDF, ADF, CP, and moisture in forages (Norris et al. 1976; Marten et al. 1983; Windham et al. 1987) and even glucose, arabinose and pectin in forages have been determined by NIRS (Fairbrother and Brink 1990). The most attractive features of NIR quantification are: speed of analysis, low labor costs, simple sample preparation, nondestructive analysis of the sample, and pollution-free analysis (Williams 1987). The chief disadvantages of the method are instrumentation requirements, dependence on calibration procedures, complexity in the choice of data treatment, and low sensitivity for minor constituents (Norris 1989).

Near infrared reflectance spectroscopy is an instrumental method for rapidly and reproducibly measuring the chemical composition of samples with little or no sample preparation (Norris 1989). It is based on the fact that each of the major chemical components of a sample has near infrared absorption properties which can be used to differentiate one component from the others. A near infrared instrument detects electronic signals diffusely reflected from a sample surface that is irradiated by the instrument with light of many wavelengths (Williams 1987). The signals at wavelengths specific to the constituents to be determined are amplified and translated by the instrument into composition data such as protein or moisture. The instrument compares all the signals received from the sample with an internal reference standard, usually ceramic or sand-blasted gold, that enables it to distinguish among samples of different composition.

The most time-consuming step in use of NIRS is calibration and this step has been an obstacle to overall acceptance of the procedure (Williams 1987). Calibration involves using a set of samples to develop a mathematical relationship between spectra generated by the instrument and values obtained from a laboratory reference method (Windham et al. 1989). Calibrations range from empirical, where the mathematical relationship is unknown and must be estimated from a set of samples, to analytical, where the form of the mathematical relationship is known ahead of time (Windham et al. 1989). All factors affecting the NIR spectra must be represented in the calibration set. This includes factors such as, sample physical and chemical characteristics, method of sample preservation and processing, and instrument and sample environment (temperature and humidity) (Williams 1987; Abrams 1989; Windham et al. 1989).

Nutritive value of moldy hay

Important factors that determine nutritive value of hay, indeed any feed, include intake, digestion and metabolism. Of these, intake is the most important because it determines what ultimately will be available to the animal for different physiological processes. Minson and Wilson (1994) reviewed chemical and physical characteristics that affect intake of a forage. Minerals, nitrogen, fibre, rate of digestion, digestibility, and other plant anatomical factors, all singly or in combination affect intake. Non-nutritive factors such as the presence of fungal biomass in hay also should be considered together with these characteristics. There is limited literature on effects of fungal biomass in moldy hay on intake, nutrient digestion and animal performance.

Miller et al. (1967) stored alfalfa forage at different DM contents ranging from 41.5 to 73.8%. On subsequent feeding to steers, hay intake was not influenced by initial DM at baling. It is important to note that DM contents at which forages in this study were stored are low and optimum for extensive accumulation of fungal biomass, even though Miller et al. (1967) reported molding only at the lower DM contents. More recent studies with moldy alfalfa hay (Mathison et al. 1986; Wittenberg and Moshtaghi-Nia 1990), alfalfa-brome mixtures (Mathison et al. 1986), and grass hay (Smith et al. 1989) have since confirmed that mold in hay does not affect hay intake.

Nutrient digestibility has been reported to be lower when moldy hay is fed to animals. Digestibilities of DM, CP, and energy were lower when steers (Miller et al. 1967) or lambs (Wittenberg and Moshtaghi-Nia 1990) were fed moldy hay. An increase in fibre digestibility due to feeding moldy hay was reported by Mohanty et al. (1969) and Smith et al. (1989). This increase in fibre digestibility was attributed to the action of fungi on de-lignifying fibre, thereby making it more available to rumen microorganisms. In sheep, feeding moldy hay decreased N balance, and this was associated with a slightly decreased N intake and a severely decreased amount of N digested combined with increased excretion of N in urine (Smith et al. 1989).

Another important consideration is how feeding moldy hay affects the rumen environment. A higher rumen pH was reported when steers were fed moldy hay and this higher pH was associated with the higher pH of moldy hay (Mohanty et al. 1969; Voelker and Mohanty, 1969). Feeding moldy hay also resulted in a lower total volatile fatty acids concentration and a wider acetic to propionic acid ratio, an effect that was attributed to low soluble carbohydrate content in moldy hay. Counts of total protozoa in the rumen were not affected by feeding moldy alfalfa hay although there were some differences in individual genera of protozoa between dairy cows fed moldy and nonmoldy hay (Mohanty et al. 1969; Voelker and Mohanty 1969).

MANUSCRIPT I: Change in forage constituents during storage of alfalfa forage baled and stored at varying dry matter contents

ABSTRACT

The purpose of this study was to evaluate the effect of DM content at baling on pattern of forage constituent change and on DM and nutrient losses during storage. Alfalfa forage, baled at 64.1-66.2, 71.9-73.2 and 75.4-77.4% DM, was designated Low, Medium and High DM treatments, respectively. Hay generated from each treatment was stored in a pole structure as two, 74 bale stacks per treatment using a bale wagon. Core samples were collected from bales on day 1, 4, 7, 14, 21 and 60 of storage. Bale temperature was monitored for 8 bales per treatment daily for the 35 days of storage and then on day 45, 50 and 60. Peak storage temperature reached was influenced ($P<0.05$) by forage DM at baling; the highest peak temperature being associated with Low DM hay. Dry matter losses also were higher ($P<0.05$) in Low DM hay relative to either Medium or High DM hays averaging, 10.1, 4.5 and 3.0%, respectively. Dry matter, NDF, ADF, ADIN and glucosamine contents increased in all forage treatments during storage and extent of increase was more ($P<0.05$) in Low DM hay relative to High DM hay. Cellulose, lignin and CP concentrations followed a similar pattern during storage for hay baled and stored at the three DM contents. Water-soluble carbohydrate content loss was higher ($P<0.05$) in Low DM hay relative to High DM hay. Forage DM content at baling will influence stack temperatures and extent of nutrient change during storage. Most changes in constituents of low DM hay occurred within the first 14 days of storage, a period within which peak temperatures were recorded in all forages. The study also showed that alfalfa forage can be stored at a DM content of 76%, which is less than normally recommended, with minimal nutrient change and loss.

INTRODUCTION

Two important factors that affect hay quality are forage DM content at baling and storage temperatures attained due to the activity of microorganisms, principally fungi, during storage (Gregory et al. 1963; Kaspersson et al. 1984). Baling forage at a low DM content results in DM loss in storage, primarily loss of non-structural carbohydrates (Kaspersson et al. 1984). As a result, cell wall, lignin and ash concentrations increase during storage (Miller et al. 1967; Cherney et al. 1987; Rotz and Abrams 1988). Heating during storage can cause an increase in bound nitrogen in the form of ADIN through chemical polymerization of sugars with amino acids (Yu and Veira 1977). Changes in forage constituent concentrations and heating result from the growth and activity of fungi and bacteria during storage. The build-up of fungal biomass in hay during storage contributes to dustiness of the forage due to spores and mycelia and an accumulation of poorly defined secondary metabolites, which together with constituent changes, result in the poor quality that is associated with moldy hay.

Significant storage losses of DM and CP due to baling forage at a low DM have been reported (Collins et al. 1987; Rotz and Abrams 1988; Wittenberg and Moshtaghi-Nia 1990). While the effects of DM at baling on long-term nutrient changes and loss, and heating in storage are well documented, there is a need to evaluate and characterize the rate at which these changes occur during storage and when changes during storage occur.

The current recommendation is that, to reduce storage DM and CP losses, forage should be stored at 80-85% DM, but at such high DM contents, field DM losses are likely to be quite high. Therefore, forage research should continuously aim at finding ways to store low DM forage, allowing reduced field losses during harvest. Leaves, the most nutritious part of forage, are subject to greater loss during harvest and handling of high DM forage. Minimum storage DM contents are not likely to be the same for different areas due to different environmental conditions nor are they likely to be the same for different forages. Also, strategies to control or eliminate development of storage fungi in low DM forages require a better understanding of events that occur during storage.

The purpose of this study was to identify changes in forage constituents during storage associated with DM content at baling. By monitoring daily temperature during storage, the effect of DM at baling on heating of forage during storage also was evaluated.

MATERIALS AND METHODS

First cut alfalfa forage (cv. Arrow) from a 15 ha weed-free stand was used for this study. The stand was cut at 30% bloom using a John Deere mower-conditioner (2.74 m swath width) into 34 swaths. Swaths were randomly assigned to three treatments. Swaths on the edge of the field were not used so as to reduce border effects. The whole field was cut on the same day, July 22/91. Forage was left to wilt in the field for 48 hours before baling and, during wilting, DM was monitored using a microwave oven. Drying conditions were good with maximum temperatures averaging 25.5°C for the 4 days during forage wilting and approximately 14 h of sunshine per d, with no precipitation (Climatological data, Glenlea Research Station; Environment Canada).

Forage was baled (John Deere 336 baler) into small square bales at DM contents of 64.1-66.2, 71.9-73.2 and 75.4-77.4% and designated as Low, Medium and High DM forage, respectively. Low DM forage was baled on July 24 /91 starting at 1:00 PM and Medium DM forage was baled the following day starting at 12:00 PM. The High DM forage treatment was baled on the same day as Medium DM forage, at approximately 3:15 PM. High DM forage was baled at a lower DM content than intended due to a forecast for rain in the evening of the same day. Two, 74 bale stacks for each forage treatment were made using a bale wagon (John Deere 1037) with the fourth layer of each stack tied. Stacks were stored in a pole shed and were placed immediately adjacent to each other with no space between them.

Sample collection

Four bales were removed from inside the stack for measurement of temperature, DM and nutrient losses. The bales were core-sampled (six cores per bale, Penn state core sampler), weighed and tagged. Thermocouple wires were inserted into these bales after which the bales were returned to their original position within the stack, which was a minimum of one bale width from the stack exterior. The bale temperatures were read daily for 35 days, and then on days 45, 50 and 60 using a Trendicator (Model 400A; Doric Scientific Div., Emerson Electric Co. San Diego, CA) which was calibrated before use by reading off thermocouple wires maintained in water at known temperatures. All thermocouple wires were tested for function before the beginning of the study. The bales were re-weighed and core-sampled 60 d post-storage. Cores from each bale were composited at stacking and post-storage, and stored (-20°C) until analysis for CP, NDF, ADF and ADIN.

Two other bales from each stack, located one bale length from the stack exterior were selected to monitor nutrient changes during storage. Each bale was core sampled (one core per bale) at different sites on days 1, 4, 7, 14, 21, 35 and 60. Samples were stored (-20°C) until analysis for CP, NDF, ADF, ADIN, water-soluble carbohydrate, cellulose, lignin and glucosamine.

Chemical analysis

Dry matter content of samples was determined by freeze-drying. Samples were analyzed for N content by the Micro-Kjeldahl method (Method 47.023, Association of Official Analytical Chemists (AOAC) 1984). Neutral detergent fibre , ADF, cellulose and lignin were determined by sequential analysis (Robertson and Van Soest 1981). Acid detergent insoluble nitrogen was determined on ADF residues (Goering and Van Soest 1970). Fungal biomass was estimated as the amount of glucosamine using the procedure described by Wittenberg et al. (1989). Water-soluble carbohydrate content of forages was determined following extraction in 80% (v/v) ethanol using the procedure described by Jermyn (1975).

Statistical analysis

Effect of treatment at baling on DM and nutrient losses, bale weights into and out of storage, and temperature were analyzed by one-way analysis of variance using general linear models (Statistical Analysis Systems (SAS) 1985). Two of the 8 thermocouple wires in the Low forage DM treatment stopped functioning after 5 days in storage. Forage constituents data were analyzed as a split plot and changes in constituent concentration were compared relative to d 1 using single degree of freedom contrasts.

Upon measuring DM content of bales on day of baling, one bale intended as a Medium DM forage treatment was found to be lower in DM content than the rest of Medium DM bales. This bale was then treated as a Low DM treatment, resulting in n=5 bales for Low forage DM treatment and n=3 for Medium DM forage. These treatment re-assignments are a reflection of uneven drying of forage while in the swath.

RESULTS AND DISCUSSION

Heating in forage treatments, as assessed by temperature, began immediately following baling. Temperature rose in all forages, reaching a transient peak two days into storage (Fig. 4). At this peak, temperature of Low DM forage was higher ($P<0.05$) than that of Medium DM forage, with the lowest ($P<0.05$) peak temperature observed for the High forage DM treatment (Table 3). Temperatures began to rise again by day 4 in all forage treatments until a second peak was reached. The time to reach this second peak was similar ($P>0.05$) among forage treatments and was about 9 days in storage (Table 3). Maximum peak temperatures attained for the second peak were influenced by forage DM, the lower the DM content, the higher the maximum temperature (Table 3). Persistence of high temperatures was most evident in Low DM forage as observed from number of days above 40 or 50°C. Temperatures in High DM forage never reached 50°C during storage (Table 3).

These results are in agreement with other work with stored forages, which show a positive linear relationship between moisture content at baling and temperature during storage (Miller et al. 1967; Weeks et al. 1975; Cherney et al. 1987).

The two temperature peaks which were observed in this study are characteristic of heating in stored forages and have been described during storage of low DM grass forages (Gregory et al. 1963; Breton and Zwaenepoel 1991). The first peak has been attributed to plant cellular respiration while the higher and more persistent second peak is due to respiration of rapidly developing fungi.

More than 80% of the moisture loss in Low and Medium DM forages occurred in the first 14 days of storage, while 58.6% of the moisture was lost from High DM forage during the same period (Table 4). Moisture loss in the first 4 days of storage accounted for 20.5, 21.0 and 6.3% of the total moisture loss in Low, Medium and High DM forages, respectively. The first 4 days of storage encompass a period when the first temperature peak was reached in all forages. The bulk of moisture loss, 59.2, 64 and 51.6% of total moisture loss for Low, Medium and High DM forages, respectively, occurred between days 4 to 14 of storage, which also happens to be the period in which temperatures in all forages were the highest (Fig. 4). Therefore, the high temperatures during this period were responsible for rapid moisture loss. Since stack arrangement was similar to that used commercially, with the exception that stacks were one layer shorter, it is anticipated that the same would be experienced in commercial stacks.

Dry matter losses during storage were 10.1, 4.5 and 3% for Low, Medium and High DM forages, respectively, and loss was higher ($P<0.05$) in Low DM forage relative to Medium and High DM forages (Table 5). Other studies storing alfalfa forage at 80-89% and 65-75% reported losses of 10.9 and 4.5%, respectively (Rotz and Abrams 1988). Most of DM lost during storage is suggested to be non-structural carbohydrate which is respired to carbon dioxide and water (Kaspersson et al. 1984).

The percent increase in CP concentration during storage was 8.1, 9.7 and 13.3%, for Low, Medium and High DM forages, respectively (Table 4). These increases in CP concentration were not different (P>0.05) among forage treatments but it is important to note that the increases start to occur within the first 14 days of storage. Crude protein retention in Low DM forage during storage was 97.4% and was lower (P<0.05) relative to either Medium or High DM forages (Table 5). Other studies on hay storage also have reported little or no change in CP content during storage (Collins et al. 1987; Rotz and Abrams 1988; Alhadhrami et al. 1993).

Acid detergent insoluble nitrogen has been used as an indicator of heat damage and has been shown to be proportional to the degree of heating (Yu and Veira 1977; Collins et al. 1987). Yu and Veira (1977) showed that heating raised the ADIN content of alfalfa haylage (47% DM) and that the increase in ADIN was correlated with the degree-days above 60°C. Acid detergent insoluble nitrogen content increased in all forage treatments during storage and 41% of the increase in ADIN in Low DM forage occurred in the first 14 days of storage (Table 4). In Medium and High DM forages, increases in ADIN of 57.2 and 55.6%, respectively, were observed during this period. It is also important to note a steady increase in ADIN concentration occurred in Low and Medium DM forages, even after peak temperatures were reached. Acid detergent insoluble N content was expected to increase around the period when temperatures were highest, with no further increase in the later part of storage. It is possible that temperature alone is not necessarily a good indicator of when and how changes in ADIN concentration begin to occur in storage.

Neutral detergent fibre content in forages increased by 13.2, 3.6 and 1.6% for Low, Medium and High DM forage treatments, respectively, by the end of storage (Table 6). Approximately 70% of the increase in NDF concentration occurred within the first 14 days of storage in Low DM forage compared to 25% in Medium DM forage during the same period. Increase in High DM forage was minimal throughout storage. The increase in NDF concentration in forages also coincides with the period in which the highest temperatures were reached (Fig. 4). Over the whole storage period increase in NDF content was higher ($P<0.05$) in Low DM forage relative to High DM forage.

Acid detergent fibre content increased by 16.3, 9.3 and 3.1% in Low, Medium and High DM forages, respectively, by the end of storage (Table 6). Similar to NDF, 90% of the increase in ADF concentration in Low DM forage occurred in the first 14 days of storage compared to a 55% increase in Medium DM forage. There was hardly any increase in ADF concentration in High DM forage throughout storage. This again suggests that whatever changes were occurring are related to microbial activity and temperature, which reaches a peak within this period. Changes in ADF concentration in Low and Medium DM forages were minimal after the first 14 days.

The increasing concentrations of NDF, ADF and ADIN with decreasing DM content at baling have been reported in many studies (Miller et al. 1967; Cherney et al. 1987; Collins et al. 1987; Alhadrahrami et al. 1993). Differences in results among studies are in the degree rather than trend and can be explained by storage conditions. Miller et al. (1967) suggested that the increase in fibre fractions, namely NDF and ADF, during storage represents a percent replacement, rather than a true increase, and is primarily due to loss of non-fibre constituents with little or no loss of fibre. These changes should also be explained in conjuction with other changes that occurred during storage, including changes in fungal counts, species and temperature. Fungal biomass might contribute to increased NDF and ADF concentrations during storage. However, in this study, change in non-fibre constituents and increase in fungal biomass still do not fully account for the increase in fibre constituents (Table 5). It may be that the constituents measured as NDF or ADF before and after storage may not be the same.

The first week of storage was marked by a rapid decline in water-soluble carbohydrate in Low DM forage (Table 6). Approximately, a 60% decline in soluble carbohydrate concentration occurred in the first 14 days in Low DM forage compared to 50 and 38% decline in Medium and High DM forages, respectively, during the same period. The early storage period is marked by high counts of bacteria and some field fungi, followed by storage fungi later in storage (Manuscript II; Kaspersson et al.1984). Since most of the water-soluble carbohydrates were depleted early in storage, bacteria and some field fungi were probably responsible for this early use. It should also be noted that the early depletion of soluble carbohydrate coincided with the rapid increase in temperatures prior to the second temperature peak. Water-soluble carbohydrate concentrations were maintained after the second peak temperatures were reached, suggesting low usage of this substrate by storage fungi. Loss in water-soluble carbohydrate content was higher ($P<0.05$) in Low DM forage relative to High DM forage when the whole storage period is considered.

The cellulose concentration of forage treatments showed relatively small increases of 11.6, 12.1 and 5.7% in Low, Medium and High DM forages, respectively, post-storage (Table 7). No differences (P>0.05) were observed in cellulose concentration due to initial DM content. Higher concentrations of cellulose at the end of storage of forages baled at different DM contents were reported (Miller et al. 1967; Cherney et al. 1987) while in other cases no differences were observed (Cherney et al. 1987; Alhadhrami et al. 1993).

Lignin content increased over time during storage in all forage treatments with percent increases of 20.5, 14.9 and 15.7% for Low, Medium and High DM forages, respectively. Over time, increases in lignin concentration showed a general upward trend. Some of the day to day variation may have been due to laboratory analysis procedures.

The forage was harvested and stored under relatively good drying conditions with no exposure to precipitation. The wilting period did not influence fungal biomass concentrations at the beginning of storage (Table 7). If forage is left in the swath for a long time under humid conditions or is rained on while in the swath, fungal biomass concentrations can increase due to accumulation of field fungi (Wittenberg and Moshtaghi-Nia 1990). Fungal biomass concentration was low in all forages in the first 7 days of storage as indicated by glucosamine concentration (Table 7). Two weeks into storage, following the second temperature peak, fungal biomass concentrations increased in all forages and the increase was greater (P<0.05) in Low DM forage relative to High DM forage. Fungal biomass did not increase any further after the initial 14 day increase. The low initial glucosamine content in all forages in the first 7 days of storage suggests that either there were low counts of filamentous fungi during this period or that the main fungal species during this time may have been yeasts.

CONCLUSION

This study suggests that the greatest change in forage constituents occurs within the first 14 days of hay storage. Changes appear to be a function of temperature and fungal growth. By day 14, bales in all three forage treatments had achieved "safe" DM contents for storage. After this point fungal growth appears to be minimal as determined by glucosamine concentration and temperature. The study also suggests that storage of alfalfa hay at DM contents as low as 75.4-77.4% will reduce water-soluble carbohydrate concentrations, with little adverse change in crude protein and structural carbohydrate concentrations. Some increase fungal biomass concentration was evident in hay baled at 75.4-77.4% DM, however, the amount of fungal biomass was less than for forage baled and stored at lower DM contents.

Figure 4. Mean bale temperatures in the initial 35 days during storage of alfalfa forage baled and stored at three DM contents of 64.1-66.2% (Low), 71.9-73.2% (Medium) and 75.4-77.4% (High). Temperatures were recorded at 12:00.

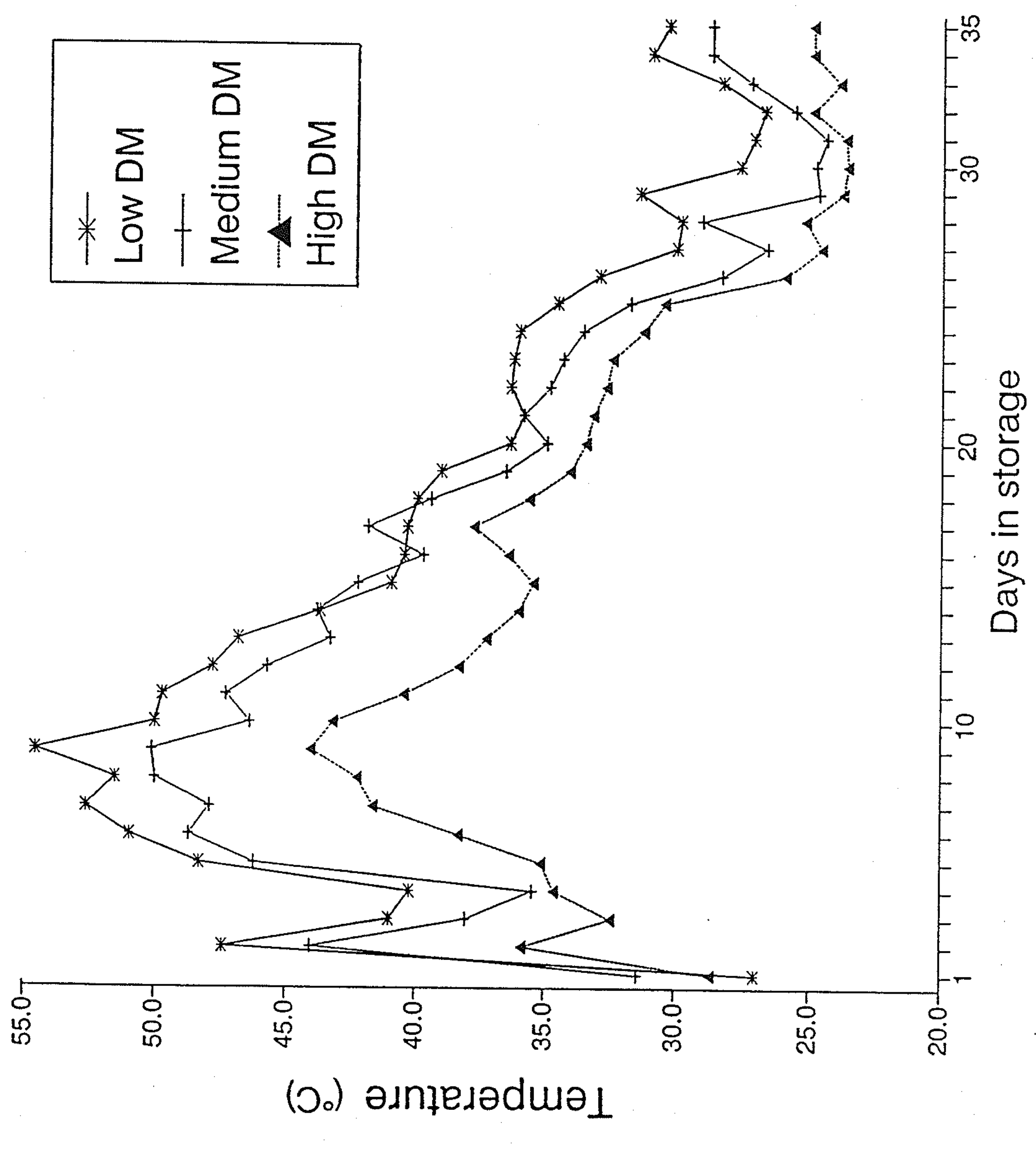

Low DM
Medium DM
High DM
Temperature (°C)
55.0
50.0
45.0
40.0
35.0
30.0
25.0
20.0
Days in storage
1
10
20
30
35

TABLE 3. Storage temperatures in alfalfa forage baled and stored at three DM contents[z]

	Forage dry matter					
	Low		Medium		High	
	Mean	SE	Mean	SE	Mean	SE
n	6		8		8	
First peak temperature (°C)	47.8a	0.8	44.0b	0.7	35.8c	0.7
Second peak temperature(°C)	54.5a	0.8	50.1b	0.7	44.0c	0.7
Days to reach maximum	8.7	0.8	9.0	0.7	8.5	0.7
Days over 40°C	14.7a	0.8	13.6a	0.7	5.0b	0.7
Days over 50°C	6.2a	0.7	1.9b	0.7	0.0b	0.6

[z] Dry matter content at baling averaged 65.3, 72.4 and 76.3% for Low, Medium and High DM forages, respectively.

a-cMeans in the same row followed by a different letter differ, $P<0.05$.

TABLE 4. Changes in dry matter, crude protein and acid detergent insoluble N during storage of alfalfa forage baled at three dry matter contents^{X,Y}

	Forage dry matter							
	Low (L)		Medium (M)		High (H)		Comparison^{Z}	
Day	Mean	SE	Mean	SE	Mean	SE	LvsM	LvsH
				Dry matter, %				
1	65.3	0.5	72.4	0.7	76.3	0.6		
4	69.5	1.1	75.5	1.4	77.0	1.1	NS	*
7	71.1	1.2	76.5	1.6	77.0	1.3	*	*
14	81.6	1.8	84.9	2.4	82.8	1.9	*	**
21	82.1	0.8	85.3	1.1	85.8	0.9	*	**
60	85.8	0.4	87.1	0.6	87.4	0.5	*	**
				Crude protein, %DM				
1	18.6	0.3	18.5	0.4	17.3	0.3		
4	18.6	0.3	18.5	0.4	18.5	0.4	NS	NS
7	19.1	0.3	20.0	0.4	18.3	0.4	NS	NS
14	19.4	0.6	20.5	0.7	18.5	0.6	NS	NS
21	18.9	0.2	18.9	0.3	18.3	0.3	NS	NS
60	20.1	0.3	20.3	0.3	19.6	0.4	NS	NS
				Acid detergent insoluble N, %total N				
1	4.6	0.1	4.3	0.2	4.2	0.2		
4	5.2	0.2	5.6	0.3	4.4	0.2	NS	NS
7	5.5	0.1	5.1	0.2	4.8	0.1	NS	NS
14	6.2	0.3	5.5	0.4	4.7	0.3	NS	NS
21	7.6	0.1	5.9	0.1	5.3	0.1	NS	*
60	8.5	0.2	6.4	0.3	5.1	0.3	NS	*

^{X}Dry matter content at baling averaged 65.3, 72.4 and 76.3% for Low, Medium and High DM forages, respectively.

^{Y}Least square means, n=5 for Low; n=3 for Medium and n=4 for High DM forage.

^{Z}Concentration change relative to day 1 for treatments being compared.

NS not significantly different (P>0.05); * significantly different (P<0.05); **(P<0.001)

TABLE 5. Bale weights into and out of storage, DM and nutrient losses in alfalfa forage baled at three DM contents[Y]

	Forage Treatment			
	Low	Medium	High	SE
Bale weight, kg DM				
Into storage	24.6a	24.4a	21.0b	0. 44
After storage	22.1ab	23.3a	20.4b	0.40
DM and nutrient loss, %[Z]				
Dry matter	11.2a	4.7b	3.2b	0.37
Crude protein	2.6a	-4.6b	-3.5b	1.07
Neutral detergent fibre	-10.7	-11.9	-7.6	0.91
Acid detergent fibre	-9.5a	-1.2b	-2.4b	0.86

[Y] Dry matter content at baling averaged 65.3, 72.4 and 76.3% for Low, Medium and High DM forages, respectively.

a-c Means in the same row followed by a different letter differ ($P<0.05$).

[Z] Nutrient loss (%)=100-((wt out/wt in)x100).

TABLE 6. Changes in neutral detergent fibre, acid detergent fibre and water soluble carbohydrate during storage of alfalfa forage stored at three DM contents^{X,Y}

	Forage dry matter							
	Low (L)		Medium (M)		High (H)		Comparison^{Z}	
Day	Mean	SE	Mean	SE	Mean	SE	LvsM	LvsH
	Neutral detergent fibre, %DM							
1	44.6	0.8	45.1	1.1	44.7	0.9		
4	42.7	1.0	42.2	1.4	43.7	1.1	NS	NS
7	49.9	1.0	44.8	1.3	44.6	1.0	NS	*
14	48.5	1.0	45.5	1.4	46.3	1.1	NS	NS
21	48.2	0.7	46.5	0.9	45.7	0.8	NS	NS
60	50.5	0.8	46.7	1.0	45.4	0.8	NS	*
	Acid detergent fibre, %DM							
1	32.6	0.5	33.3	0.7	34.6	0.6		
4	34.4	0.8	32.7	1.1	32.3	0.9	NS	*
7	35.2	0.6	34.9	0.8	33.1	0.7	NS	*
14	37.5	1.1	35.4	1.5	34.9	1.2	NS	*
21	37.5	0.8	36.9	1.1	35.1	0.9	NS	*
60	37.9	0.7	37.1	0.9	34.7	0.7	NS	*
	Water-soluble carbohydrate, %DM							
1	6.5	0.3	5.6	0.4	6.3	0.4		
4	5.1	0.3	5.5	0.3	6.0	0.3	NS	*
7	2.6	0.3	3.1	0.5	4.3	0.4	NS	*
14	2.6	0.2	2.8	0.2	3.9	0.2	NS	*
21	3.1	0.3	3.1	0.4	3.3	0.3	NS	NS
60	2.6	0.2	2.9	0.3	3.3	0.3	NS	*

[X] Dry matter contents at baling averaged 65.3, 72.4 and 76.3% for Low, Medium and High DM forages, respectively.

[Y] Least square means, n=5 for Low, n=3 for Medium and n=4 for High.

[Z] Concentration change relative to day 1 for treatments being compared.

NS not significantly different (P>0.05); *significantly different (P<0.05).

Table 7. Changes in cellulose, lignin and glucosamine during storage of alfalfa forage stored at three DM contents[X,Y]

	Forage dry matter							
	Low (L)		Medium (M)		High (H)		Comparison[Z]	
Day	Mean	SE	Mean	SE	Mean	SE	LvsM	LvsH
			Cellulose, %DM					
1	24.2	0.5	23.9	0.7	24.5	0.6		
4	24.6	0.5	24.8	0.7	24.0	0.5	NS	NS
7	25.1	0.4	23.5	0.5	24.8	0.4	NS	NS
14	26.6	0.6	26.2	0.9	24.9	0.7	NS	NS
21	26.3	0.5	24.3	0.7	24.5	0.6	NS	NS
60	27.0	0.6	26.8	0.8	25.9	0.6	NS	NS
			Lignin, %DM					
1	7.8	0.2	7.4	0.3	7.0	0.2		
4	7.6	0.3	7.9	0.5	7.3	0.3	NS	NS
7	8.2	0.3	6.9	0.3	7.2	0.3	NS	NS
14	9.4	0.3	8.6	0.4	8.9	0.3	NS	NS
21	9.2	0.3	8.7	0.4	8.2	0.3	NS	NS
60	9.4	0.2	8.5	0.2	8.1	0.2	NS	NS
			Glucosamine, gkg^{-1}					
1	1.6	0.04	1.6	0.06	1.5	0.04		
4	1.6	0.06	1.4	0.08	1.5	0.07	NS	NS
7	1.9	0.07	1.7	0.1	1.4	0.08	NS	*
14	3.0	0.2	2.6	0.2	2.2	0.2	NS	**
21	3.1	0.3	3.0	0.4	2.1	0.3	NS	*
60	3.4	0.2	2.9	0.2	1.9	0.2	*	**

[X] Dry matter contents at baling averaged 65.3, 72.4 and 76.3% for Low, Medium and High DM forages, respectively.

[Y] Least square means, n=5 for Low; n=3 for Medium and n=4 for High DM forage.

[Z] Concentration change relative to day 1 for treatments being compared.

NS not significantly different (P>0.05), *significantly different (P<0.05); **(P<0.001).

Manuscript II: Occurrence of fungal species in stored alfalfa forage as affected by moisture content at baling and temperature during storage

ABSTRACT

A study was conducted to evaluate the effect of moisture content at baling on fungal activity in stored alfalfa forage. Alfalfa forage was baled at DM contents of 64.1-66.2% (Low), 71.9-73.2% (Medium) and 75.4-77.4% (High) and was sampled 1, 2, 3, 4, 5, 6, 7, 8, 9, 14, 21, 35 and 60 d during storage. The data were subjected to ecological measurements which included total fungal counts, number of species, species diversity, species dominance and to correspondence and canonical correspondence analysis, following species identification. Total fungal counts, number of species, species diversity, and species dominance were not influenced ($P>0.05$) by moisture content in the early storage phase, days 1 to 8. Moisture content at baling had an influence ($P<0.05$) on total counts, number of species, and species dominance in the last phase of storage, days 9 to 60. Total fungal counts were highest in Low DM forage, and number of species highest in Medium DM forage. Species dominance was highest in High DM forage. Low forage DM was associated *Aspergillus fumigatus*, *Mucor*, *Absidia* spp., *E. nidulans*, and thermotolerant hyphomycetes. *Aspergillus repens*, *Absidia* spp., and some yeasts were more predominant in Medium and High DM forages. Moisture content and temperature were important in explaining species assemblages during storage but water-soluble carbohydrate, crude protein, and total bacteria were not. "Field" fungi, *Phoma*, *Alternaria*, *Cladosporium* spp. and most yeasts were eliminated within 8 days of storage. Conditions created in the early stages of storage will affect fungal growth in the later storage phase.

INTRODUCTION

Approximately 5.8 million hectares were used to produce about 29 million metric tonnes of tame hay in Canada in 1991 (Statistics Canada 1992). Fifty-six percent of the land was used to produce about 16.4 million metric tonnes of alfalfa and alfalfa-grass hay, with an estimated value of approximately $1 billion. Only a small fraction (0.2%) of total hay produced was exported, the rest presumably being used to feed livestock on the farm or sold within the country. One major determinant of hay price, be it for export or local trade, is hay quality, and quality evaluation takes into account nutrient composition as well as visual assessment for color and mold.

Moldy hay poses a risk to, both animals that are fed and to individuals who handle hay. Mycotic abortion and mycotic mastitis in cattle, and farmer's lung in humans have been associated with feeding and handling of moldy hay (Lacey 1975; Knudtson and Kirkbride 1992). Storage fungi in hay also are potential mycotoxin producers, which is a further source of concern. Thus, there is a need to further understand fungal growth during storage of hay. Information that is available on fungal growth during hay storage has originated from European studies with grass forages (Gregory et al. 1963; Breton and Zwaenepoel. 1991).

Such work has shown that, before harvest and during field wilting, field fungal species such as *Cladosporium*, *Alternaria*, *Fusarium* and *Phoma* predominate in grass forage (Gregory et al. 1963; Breton and Zwaenepoel 1991) although some storage species, namely, *Penicillium*, *Mucor*, *A. glaucus,* and *A. flavus* also have been isolated during wilting (Pizarro and Warboys 1980; Clevstrom and Ljunggren 1984).

Field fungi are replaced by storage fungi during storage and environmental factors such as moisture content and temperature may ultimately determine the fungal species that predominate in storage. Common storage fungi include, different species of *Aspergillus*, *Mucor* and *Absidia* (Lacey 1989; Breton and Zwaenepoel 1991). The *Aspergillus glaucus* group, mainly, *A. repens* and *A. amstelodami* predominate in forage baled at 75% DM while higher moisture contents result in dominance of thermolerant species such as *A. fumigatus*, *Humicola lanuginosa*, *A. versicolor*, and *Mucor* (Gregory et al. 1963; Lacey 1975; Kaspersson et al. 1984; Breton and Zwaenepoel 1991).

In North America, where alfalfa is used extensively to make high quality hay, very little effort has been directed towards evaluating fungal growth during storage of this forage crop. The purpose of this work was to evaluate the effect of moisture content at baling on population ecology of fungal species in alfalfa forage stored at different DM contents.

MATERIALS AND METHODS

Alfalfa forage was baled and stored at Low (64.1-66.2%), Medium (71.9-73.2%), and High (75.4-77.4%) DM contents with two, 74 bale stacks representing each forage treatment. One bale from the Medium DM forage treatment was found to be low in DM and as a result, was treated as a Low DM treatment. This resulted in n=4 for High DM bales, n=3 for Medium and n=5 for Low DM bales. Harvest and storage procedures used in this study were described earlier (Manuscript I).

Sample collection

Two bales were selected at random from each DM level-stack combination for successive sampling during storage. These bales were positioned one bale width from the stack exterior. Bales were core-sampled (one core per bale) using a Penn State core-sampler at the time of stacking and on d 2, 3, 4, 5, 6, 7, 8, 9, 14, 21, 35 and 60. Day 1 was considered to be the day of stacking. Samples were taken asceptically by disinfecting the core pipe and tip using a 70:30 ethanol:water solution between samples. Forage samples collected from bales were placed on ice and immediately transported to the laboratory.

Day 1 core sampling was done approximately 3.5 h after baling was initiated for the Low DM forage treatment. Medium and High DM hay bales were core sampled 4 h and 2.75 h after baling was initiated, respectively. Starting on the third day after stacking, samples were taken from all forage treatments at 12:00 PM. Thermocouple wires were placed into 4 bales per stack to monitor daily temperature during storage.

Sample preparation

Samples were stored at 4°C until time of processing, which was completed within 24 h of sample collection. Approximately 2 g of sample was removed from whirlpac bags using sterile forceps and placed into sterilized Kimax test tubes with lids. Fifteen ml of wash solution (5 g peptone, 3 g yeast extract and 20 ml glycerol/l) was dispensed into each tube. The tubes were then sealed, placed onto a rack and suspended horizontally in a controlled environment incubator-shaker at room temperature and shaken at 400 rpm for 5 min. The rack was then flipped and the process repeated. The wash solution was decanted and 30 ml of sterilized water added into each tube. This suspension was centrifuged at 21 xg for 10 min, the water decanted, and the process repeated 3 times.

Another 15 ml of wash solution was added again and the suspension was centrifuged at 21 xg for 5 min, and the supernatant decanted leaving 1 ml at the bottom of the tube. Tubes were then centrifuged at 1360 xg for 10 min, the remaining wash solution decanted off, leaving a pellet. Approximately 0.15 ml of glycerol was then added to each tube, the contents vortexed and placed into storage containers. This stock solution was stored at -60°C until time of plating.

Microbiological analysis

Samples were removed from the freezer and were allowed to thaw at room temperature. One ml of sterile wash solution was added to each sample and then serial dilutions made. Approximately 0.1 ml of appropriate dilutions were put onto plates in duplicate and spread using an L-shaped glass rod which was flamed before use. Three media were used, Dichloran Rose Bengal agar for enumerating filamentous fungi (King et al. 1979), Oxoid agar with cyclohexamide for enumerating bacteria and Difco agar to which penicillin and streptomycin were added, for enumerating yeasts.

Filamentous fungi and yeasts plates were incubated at 25 and 40°C for up to 5 days before they were counted. Plates for bacteria were incubated at 30 and 60°C and could be counted after 3 days. Fungi identification was based on morphological examinations (Pitt and Hocking 1985). Isolates were sent to the International Mycology Institute (Biosystematics Services, Bakeham Lane, Surrey, UK) for verification.

Statistical analysis

A complex data set resulted because fungal species occurred at different times during storage. There also was considerable variation in fungal counts and species presence or absence on a daily basis (Table 8). This required statistical analysis used to study population dynamics. The data were subjected to measurements of ecological diversity which included, the Berger-Parker dominance index and the log series α diversity index, in addition to total fungal counts and number of species.

The Berger-Parker index is a dominance measure expressing the proportional importance of the most abundant species (Magurran 1988) and is given by:

$$d = \frac{N_{max}}{N}$$

where N=total number of individuals and N_{max}=the number of individuals in the most abundant species.

The log series α diversity index attempts to describe mathematically the relationship between the number of species and the number of individuals in those species (Magurran 1988). The log series α index is obtained from:

$$\alpha = \frac{N(1 - x)}{x}$$

where: N=total number of individuals and x is estimated from the iterative solution of:

$$\frac{S}{N} = \frac{(1 - x)}{x[-\ln(1 - x)]}$$

Where S=total number of species (Magurran 1988).

Repeated measures analysis was used to determine the effects of moisture content at baling on total fungal counts, number of species, the log series α index and the Berger-

Parker index. Total counts were transformed to log(e) before analysis. Analysis was carried out using SYSTAT (Wilkinson 1988), except for the Berger-Parker index, which was done using Statistical Analysis Systems (SAS 1985) software.

The storage period was considered to consist of two phases, an early phase, which was from the day of baling (day 1) to day 8. The later storage phase was from day 9 to the end of storage. This division was based on temperature during storage. The first 8 days of storage were marked by increasing temperatures until peak temperatures were reached on about day 9 (Fig. 5).

The data for sites and fungal species also were subjected to correspondence analysis (CA) and canonical correspondence analysis (CCA) using CANOCO software (ter Braak 1987-92). For these analyses, the storage period also was divided into an early phase and a later phase, following the same rationale as for the repeated measures analyses. Environmental variables tested in CCA included moisture content, crude protein, total bacteria, water-soluble carbohydrate, and temperature.

RESULTS AND DISCUSSION

All forages began to heat immediately following stacking and reached a transient peak after two days in storage (Fig. 5). At this peak, temperature of Low DM forage was higher ($P<0.05$) than that of Medium DM forage and temperature of Medium DM forage was higher ($P<0.05$) than that in High DM forage. Temperature changes during storage were discussed in detail earlier (Manuscript I). Moisture loss in forage treatments during storage is shown in Figure 6 and has been discussed earlier (Manuscript I).

Total fungal counts varied ($P<0.05$) on a daily basis within treatments (Fig. 7).

However, during the early phase, daily variation in total fungal counts was not influenced ($P>0.05$) by moisture content or day. Moisture content had an influence ($P<0.05$) on total fungal counts after 9 days of storage, fungal counts being consistently higher in the Low DM forage relative to Medium and High DM forages. These data suggest that there is not a linear relationship between forage DM at baling and total fungal counts.

Although moisture content did not influence fungal counts, it did influence ($P=0.051$) number of fungal species during the early phase of storage, the highest number of species being observed in the Medium DM forage. There also was high ($P<0.05$) day to day variation in number of species within each treatment (Fig. 8). The dramatic fluctuations in number of species within each treatment occurred during a time when storage temperatures were rising (Fig. 5). The number of species also was influenced ($P<0.05$) by moisture content at baling during the later phase. It would seem that storage conditions for the medium DM forage were conducive to the highest number of fungal species during storage.

Species diversity, as determined by the log series α index, was not influenced ($P>0.05$) by moisture content at baling (Fig. 9). Species diversity also did not vary ($P>0.05$) on a daily basis, within each treatment in either the early or late storage phase.

The Berger-Parker index is a dominance measure since it is weighted towards the abundance of the most common species, rather than providing a measure of species richness (Magurran 1988). In the early storage phase, dominance was not influenced ($P>0.05$) by moisture content at baling or by day ($P>0.05$) within each treatment (Fig. 10). Although dominance during this period was not influenced by moisture content at baling, the predominant species were different (Table 8). Yeasts were predominant in Medium and High DM forages, while *Phoma* and *Cladosporium* spp. were predominant in Low DM forage. Storage species appeared much earlier in Low DM forage and this was most probably related to the higher temperature attained.

Moisture content at baling had an influence ($P<0.05$) on species dominance from day 9 onwards, with species dominance being consistently higher in High DM forage (Fig. 10). *Aspergillus repens* was most often dominant in Medium and High DM forages (Table 8). Unidentified thermotolerant hyphomycetes following either 25 or 40°C incubation were most often dominant in Low DM forage. Thermotolerant hyphomycetes were treated as two different species based on their incubation temperature but they could, in fact, be the same organism. However, since they were not identified, it is much safer to treat them as two different organisms.

Correspondence analysis (CA) was discussed in detail by Greenacre (1984) and ter Braak (1985). Correspondence analysis gives a unique ordination of both the sites and of the species in a site x species matrix. In the context of the current study, "site" represents any one of the three DM treatments on a specific day. The axes in a CA ordination diagram can be thought of as hypothetical environmental variables and are presumed to relate to underlying environmental variables (ter Braak 1985). The positions of sites and species points with respect to each other show a general relationship. Each species point will lie more or less "in the direction of" the site in which that species is prominent (Greenacre 1984). The distance between site points is a measure of similarity in their profiles, hence the further away sites are from each other, the more different they are in the species profiles.

The CA ordination diagrams accounted for 50% of the variation in the data set representing the whole storage period (Fig. 11). Forty-two (42.1%) percent of the variation was attributable to the horizontal axis, which separated early sites of Low, Medium and High DM forages on the positive side of the axis and later sites on the negative side, suggesting storage time is a major contributer to this axis. The early sites were associated with *Cladosporium* , *Phoma*, *Alternaria* spp., and several yeast species. Therefore, these CA ordination diagrams show a progressive extinction of field species and an increase in storage species with time (Fig. 11). The later sites were associated with *A. repens*, *Mucor* spp., *E. nidulans*, *Absidia* spp., *A. flavus*, and thermotolerant hyphomycetes.

The CA ordination diagrams for d 9 to 60 of storage also explained a high (51.6%) percentage of the variation (Fig. 12). Site separation was along the horizontal axis which accounted for 32.1% of the variation. Species associated with Low DM sites were *Absidia*, *Mucor*, *E. nidulans*, *A. fumigatus*, unidentified thermotolerant hyphomycetes, and some yeast species (Y3 and Y7). There was no clear separation of species assemblages between Medium and High DM forage sites, and these sites were associated mainly with *A. repens*, *Phoma* spp. and yeast species (Y2, Y5, and Y8).

Canonical correspondence analysis was discussed in detail by ter Braak (1986; 1987). The CCA provides an integrated description of species-environment relationships by assuming a response model that is common to all species, and the existence of a single set of environmental variables to which all species respond. The resulting ordination diagram shows the pattern of variation in community composition as accounted for by the environmental variables, and also shows approximately the distribution of species along each environmental variable. The CCA can be displayed in an ordination diagram and species and sites are represented by points while environmental variables are represented by arrows (ter Braak 1987). The arrow of an environmental variable points in the direction of maximum change of that variable across the diagram, its length being proportional to the rate of change in this direction.

The CCA ordination for the whole storage period resulted with the environmental variable "day" coinciding with the horizontal axis (Fig. 13). This variable "day" accounted for 38.1% of the variation in the data and the resulting ordination supported what was observed with CA analysis. Other environmental variables tested were crude protein, water-soluble carbohydrate content and total bacteria and they did not contribute to explaining occurrence of fungal speices.

Moisture content and temperature accounted for 15.8% of the variation in the data during the later phase of storage, with moisture content accounting for most of the variation (9.9%; Fig. 14). More Low DM sites were situated in positions of higher moisture content and more Medium and High DM sites were in situations where moisture content was relatively lower. Associations of fungal species and sites were similar to those in CA ordinations for the same period.

Many environmental factors play a role in explaining fungal species succession in stored agricultural products and among these available moisture, temperature, substrate, and presence of competing microbial species are important (Magan and Lacey 1984a;

Pitt and Hocking 1985). Fungal species have different requirements with regard to available moisture and temperature and these cannot be regarded independently. In general, organisms associated with the early storage phase have higher moisture requirements and lower temperature optima relative to organisms in the later storage phase. Field fungi grow well in the temperature range of 0-30°C (Christensen 1987; Lacey 1989).

Other environmental factors affecting mold growth during storage include pH, gaseous composition of the atmosphere, nutrient availability, and interaction with other organisms (Magan and Lacey 1984a; Pitt and Hocking 1985). Most fungal species are not affected by pH over a broad range of 3 to 8, and the optimum seems to be 5 (Pitt and Hocking 1985). In forage samples collected during storage of the current study, pH averaged 6.5 with no change over time. Fungi are capable of deriving nitrogen and energy from a wide range of sources (Pitt and Hocking 1985). Previous work indicated little change in N but loss of water-soluble carbohydrate by day 14 of storage of storage (Manuscript I). When these variables were imposed on the CCA they did not contribute to explaining species assemblages during storage.

Besides these environmental factors, fungi rarely occur in a monospecific culture in stored products but often as a group of interacting species along with bacteria, and often the conditions under which fungi form in greatest numbers are those at which they survive or compete best, not at which they grow best (Magan and Lacey 1984b; Lacey 1989). Total bacterial counts in the current study averaged 2.2×10^5, 9.1×10^4 and 3.8×10^5 cfu/g of forage DM for Low, Medium and High DM forages, respectively, and total counts were 10^2 lower in Low DM hay than in High DM hay by the end of storage (Appendix II). Generally, total bacterial counts decreased as storage progressed. Total bacterial counts, when imposed as an environmental variable, were not important in explaining fungal species assemblages during storage. This has implications with regard to use of bacterial preservatives in hay storage. Bacteria preservatives are applied to provide total counts similar to indigenous populations in forage treatments above, and their effects on hay storage have been variable. Application rates may have to be increased to observe more positive results.

The occurrence of fungal species in Low, Medium and High DM forage in the current study can be interpreted with the help of the measured environmental variables, moisture content and temperature. Moisture content and a_w are linearly related, and generally, a higher moisture content is associated with a higher a_w. Albert et al. (1989) constructed moisture sorption isotherms (MSI) for alfalfa leaves and stems. In high moisture alfalfa, a_w 0.85 corresponded with a moisture content of 23.6 and 26.2% for stems and leaves, respectively, in early cut forage, and 22.1 and 24.9%, respectively, in late cut forage.

Using the MSI by Albert et al. (1989), forages in the current study were stored at approximate a_w 0.93, 0.88 and 0.85 for Low, Medium and High DM forages, respectively. The highest temperatures attained during storage for Low, Medium and High DM forages were 54.5, 50 and 44°C, respectively.

By the time peak temperatures were reached in all forages, species which have optimum temperatures for growth of about 25°C and a_w 0.99-1.00 had been eliminated, and these were *Cladosporium*, *Alternaria* and *Phoma* spp. The later storage phase was marked by an appearance of several *Aspergillus* spp., *Mucor*, *Absidia* spp. and some yeasts. *Aspergillus* spp. are characteristic colonizers of stored products and, since they differ considerably in their requirements, the predominant species is often a good indicator of storage conditions (Lacey 1989). In Medium and High DM forages, *A. repens* was the dominant fungal species. *Aspergillus repens* thrives in agricultural products stored at 20-25% moisture (0.90a_w) which heat to about 35°C, but the optimum for growth is 25-27°C (Lacey 1989). *Emericella nidulans* and *A. fumigatus* were found in Low DM forage and certainly conditions in this forage were favourable for proliferation of these species. Both these species have the ability to grow in high temperature environments up to 50°C for *E. nidulans* and 60-65°C (with an optimum of 40-42°C) for *A. fumigatus,* and high moisture environments, 30-35% (a_w 0.97) for *E. nidulans* and 35-40% (a_w >0.98) for *A. fumigatus* (Lacey 1989).

During the later storage phase there was little difference in temperature and moisture content among forages. Therefore, it appears that unidentified constraints may be associated with appearance of different dominant species. It is speculated that this constraint may have been peak temperature.

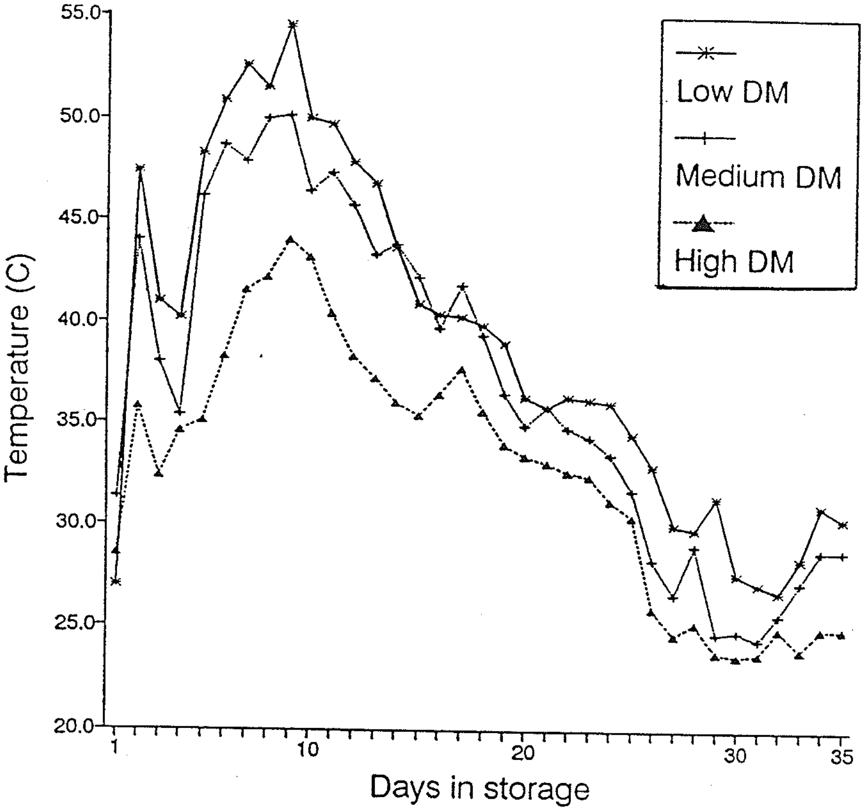
Mucor and *Absidia* spp. also occurred to a larger extent in Low DM forage which might indicate tolerance for high moisture and temperature. The most dominant fungal species in Low DM forage were unidentified thermotolerant hyphomycetes, but since they were not identified, their ecological requirements cannot be discussed with any certainty. These species seem to thrive in high moisture environments where forage can heat to high temperatures. Yeasts were predominant only in the early storage phase and most were eliminated by day 9.

CONCLUSION

Moisture content at baling was important in determining total fungal counts in forage, number of species, species diversity and species dominance. The most important fungal species after 60 d storage of alfalfa stored at Medium and High DM contents were *A. repens*, *Absidia* spp., and some yeasts. In Low DM forage there was a predominance of *E. nidulans*, *A. fumigatus*, *Absidia* spp. and unidentified thermotolerant hyphomycetes. Moisture content influences temperature during storage, and temperature during the early storage period likely influences fungal species and succession in the later phase of storage. More stable conditions in terms of fungal counts and number of species were found only after temperatures had peaked and were beginning to decrease in all forage treatments.

Figure 5. Temperature changes during the initial 35 d of storage of alfalfa forage baled at Low (64.1-66.2%), Medium (71.9-73.2%) and High (75.4-77.4%) DM contents. (Previously presented in manuscript I).

Figure 6. Moisture change during storage of alfalfa forage baled and stored at Low (64.1-66.2%), Medium (71.9-73.2%) and High (75.4-77.4%) DM contents.


55.0
50.0
45.0
40.0
35.0
30.0
25.0
20.0
Temperature (C)
1
10
20
30
35
Days in storage
Low DM
Medium DM
High DM

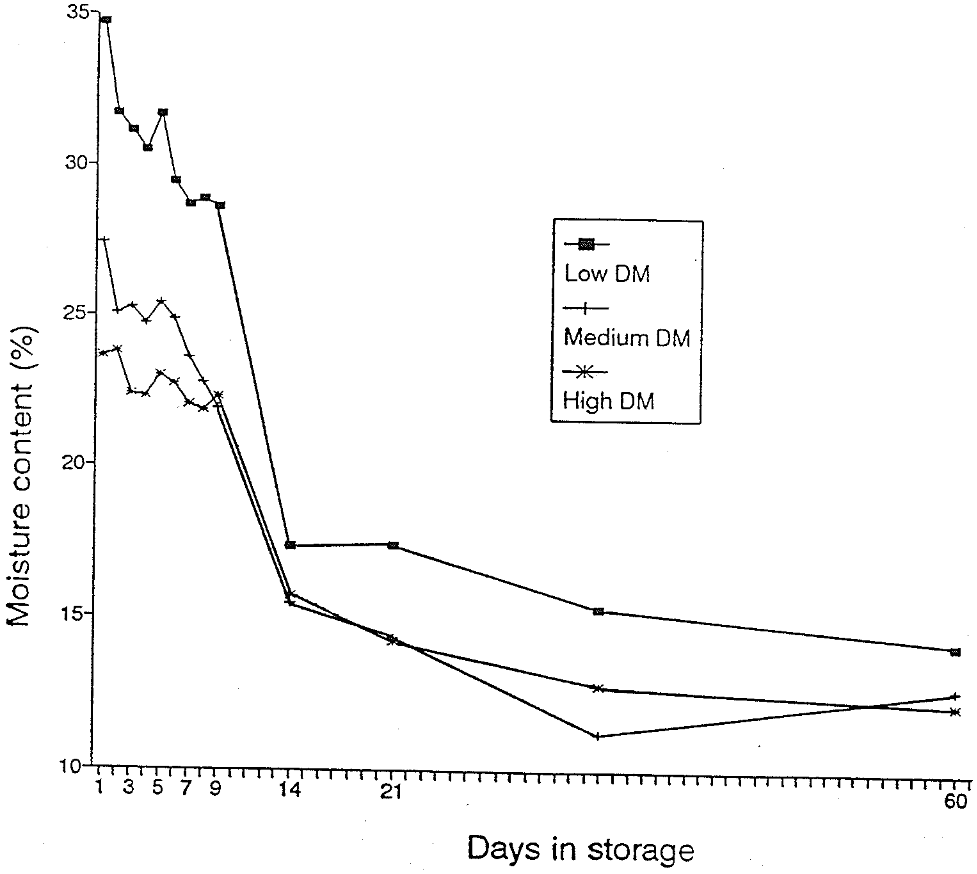
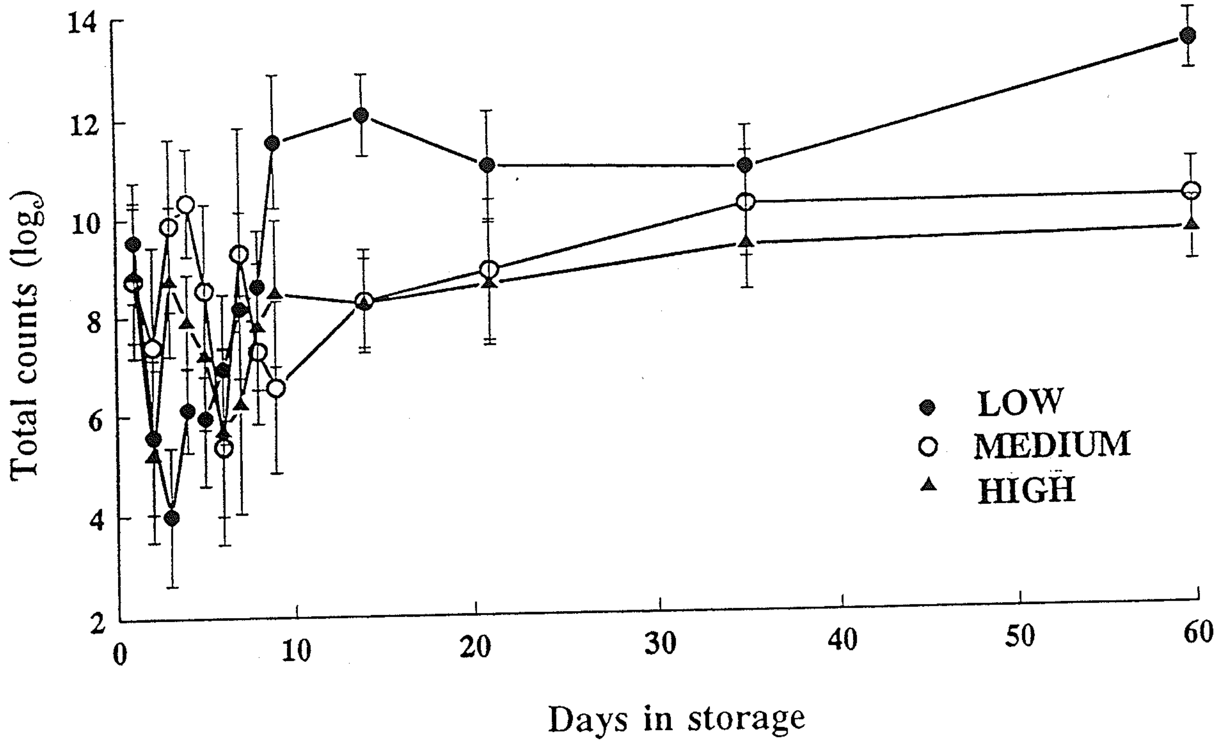

35
30
25
20
15
10
Moisture content (%)
1
3
5
7
9
14
21
60
Days in storage
Low DM
Medium DM
High DM

Figure 7. Total fungal counts during storage for alfalfa forage baled at Low (64.1-66.2%), Medium (71.9-73.2%) and High (75.4-77.4%) DM contents.

Figure 8. Number of fungal species during storage for alfalfa forage baled at Low (64.1-66.2%), Medium (71.9-73.2%) and High (75.4-77.4%) DM contents.


Total counts (log$_e$)
14
12
10
8
6
4
2
0
10
20
30
40
50
60
Days in storage
● LOW
○ MEDIUM
▲ HIGH

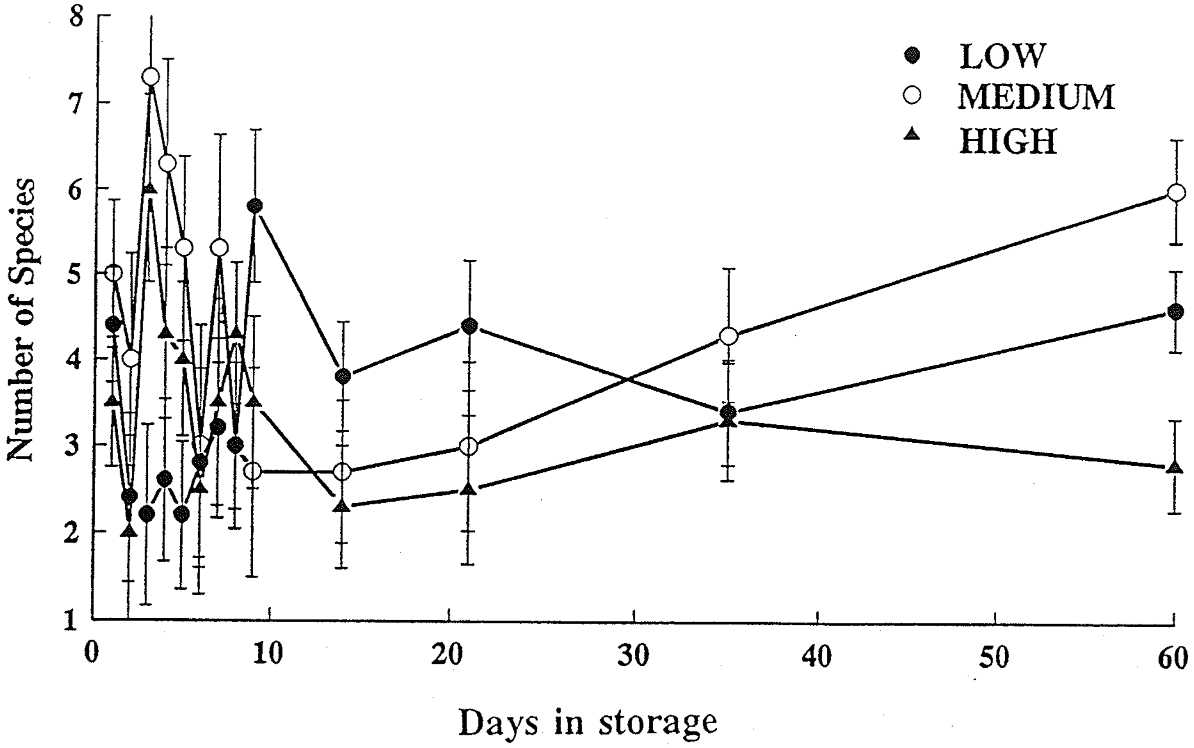
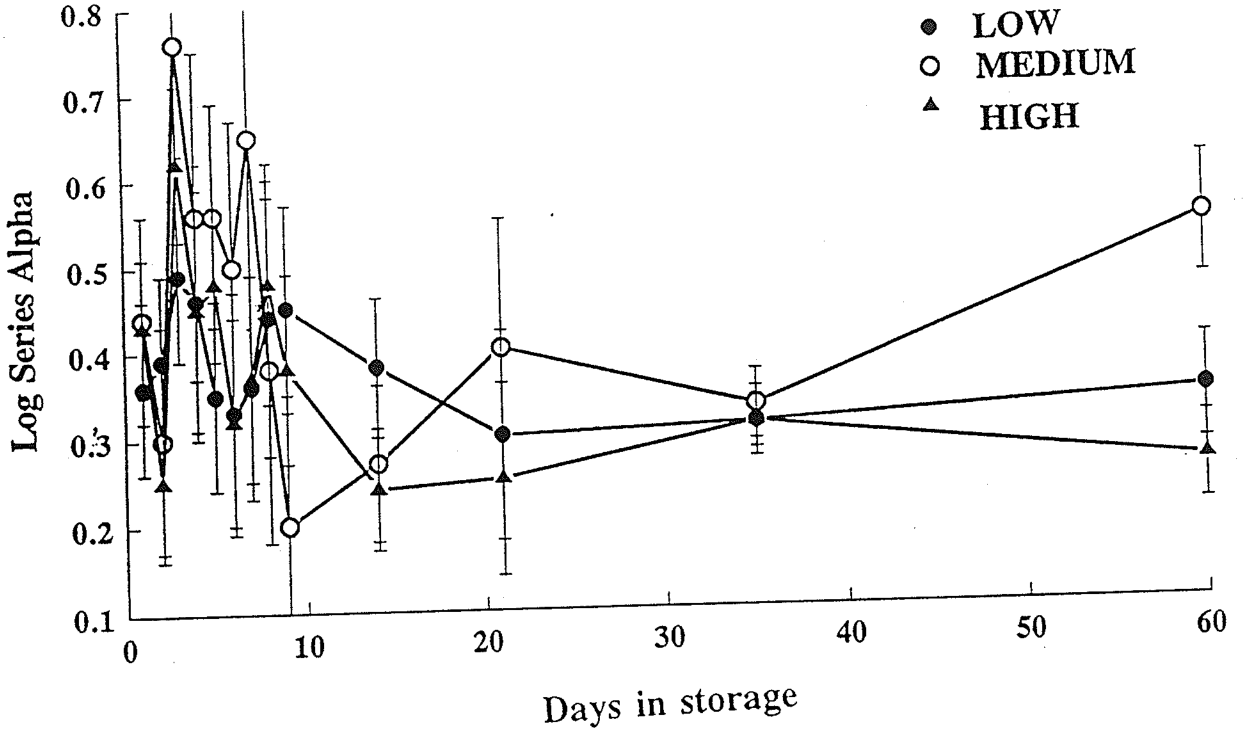

Number of Species
8
7
6
5
4
3
2
1
0
10
20
30
40
50
60
Days in storage
● LOW
○ MEDIUM
▲ HIGH

Figure 9. The log series α diversity index for fungi in alfalfa forage baled at Low (64.1-66.2%), Medium (71.9-73.2%) and High (75.4-77.4%) DM contents

Figure 10. The Berger-Parker index for fungi in alfalfa forage baled at Low (64.1-66.2%), Medium (71.9-73.2%) and High (75.4-77.4%) DM contents


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Log Series Alpha
0
10
20
30
40
50
60
Days in storage
● LOW
○ MEDIUM
▲ HIGH

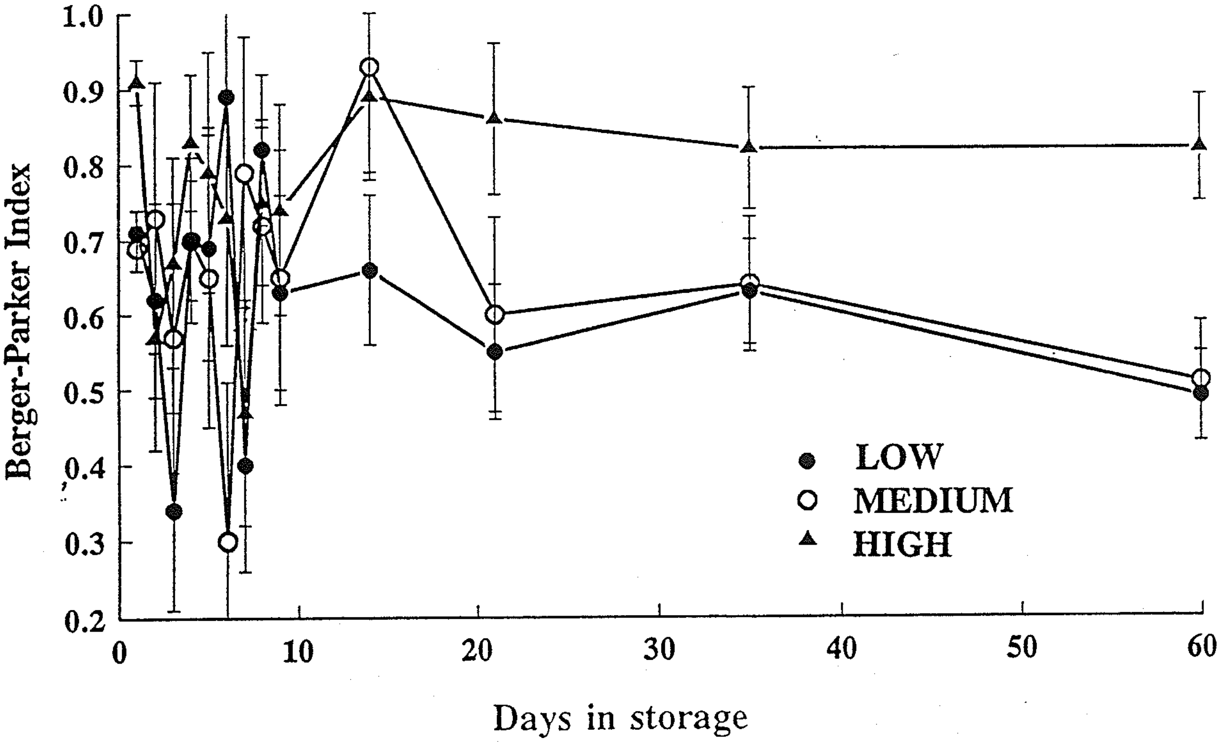
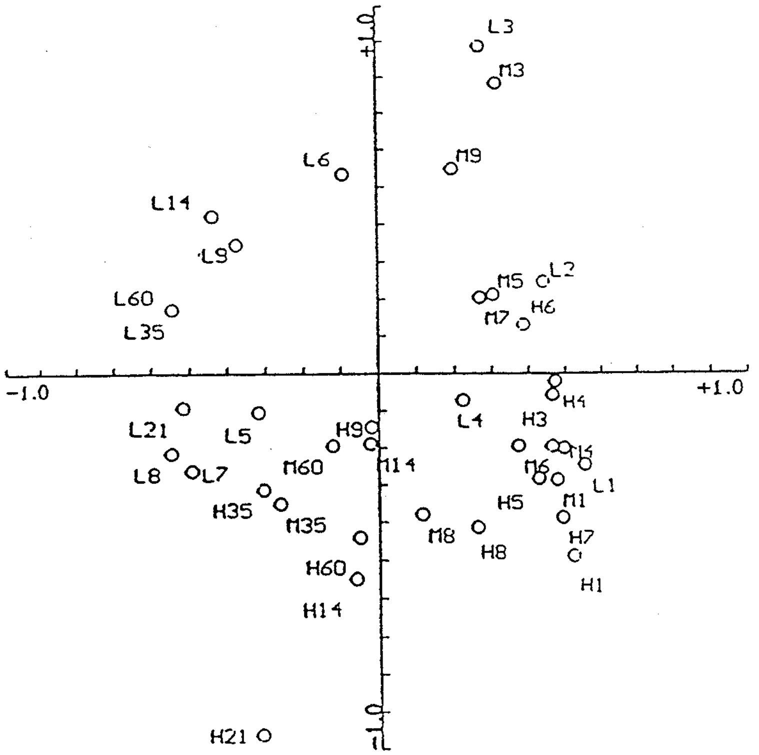

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Berger-Parker Index
0
10
20
30
40
50
60
Days in storage
● LOW
○ MEDIUM
▲ HIGH

Figure 11. The detrended CA ordination diagrams for 60 d of storage showing sites (o) and fungal species (●). The fungal species include yeasts which are numbered (Y) 1 to 8; Cla=*Cladosporium* spp., Alt=*Alternaria* spp., Pho=*Phoma* spp., Aspr=*A. repens*, Aspflv= *A. flavus*, Asp1=*E. nidulans*, red=an unidentified spp., Unk25 and Unk40=Unidentified thermotolerant hyphomycetes incubated at 25° and 40°C, respectively. Abs and Abs40=*Absidia* spp. incubated at 25 and 40°C, respectively. Muc and Muc40=*Mucor* spp. following incubation at 25°C and 40°C, respectively. The site numbering represents the forage treatment and day of sampling, e.g. L1=Low DM forage site on day 1 of storage.


+1.0
L3
M3
M9
L6
L14
L9
L2
M5
H6
M7
L60
L35
-1.0
+1.0
H4
L4
H3
L21
L5
H9
M60
H14
M4
L8
L7
M6
L1
H5
M1
H35
M35
M8
H8
H7
H60
H1
H14
-1.0
H21

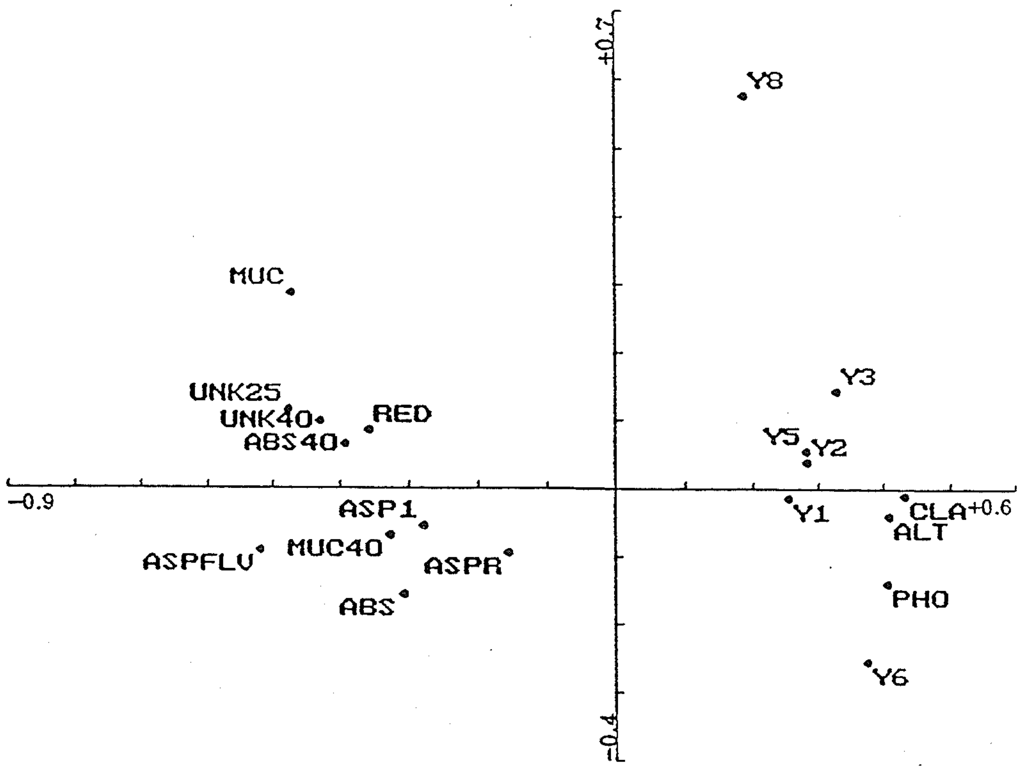

+0.7
Y8
MUC
UNK25
UNK40
RED
ABS40
Y3
Y5
Y2
-0.9
ASP1
Y1
CLA
+0.6
ALT
MUC40
ASPFLV
ASPR
ABS
PHO
Y6
-0.4

Figure 12. The CA ordination diagram for d 9 to 60 of storage showing sites (o) and fungal species (●). Fungal species and sites are defined as in Fig. 11.

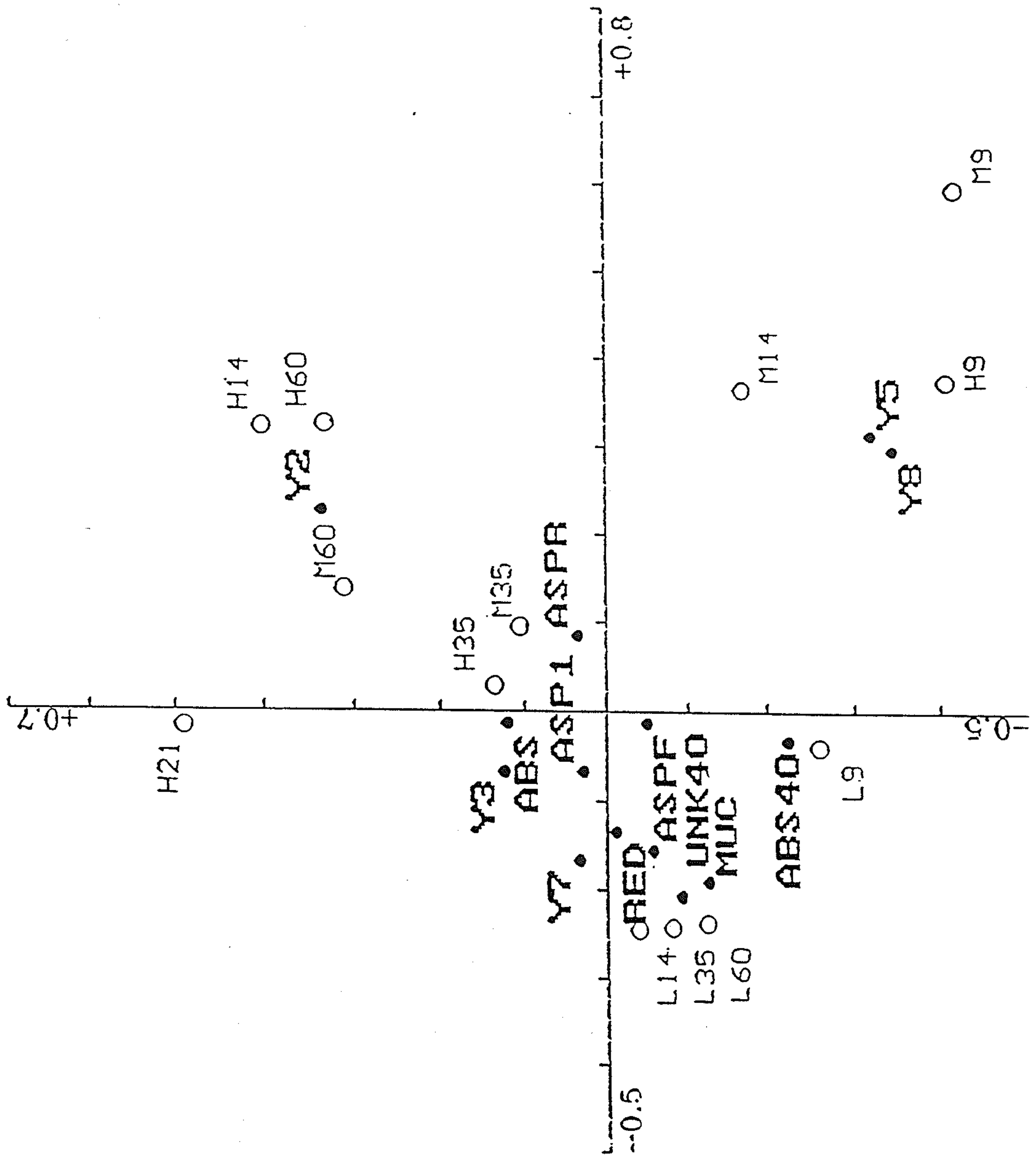

+0.8
-0.5
+0.7
-0.5
H21
H14
H60
Y2
M60
M35
H35
ASPR
ASP1
ABS
Y3
ASPF
UNK40
MUC
RED
Y7
L14
L35
L60
ABS40
L9
M14
H9
M9
Y5
Y8

Figure 13. The CCA ordination diagram for the 60 d storage period with fungal species and day (arrow) as the environmental variable. Day coincided with the horizontal axis. The species are defined as in Fig. 11.

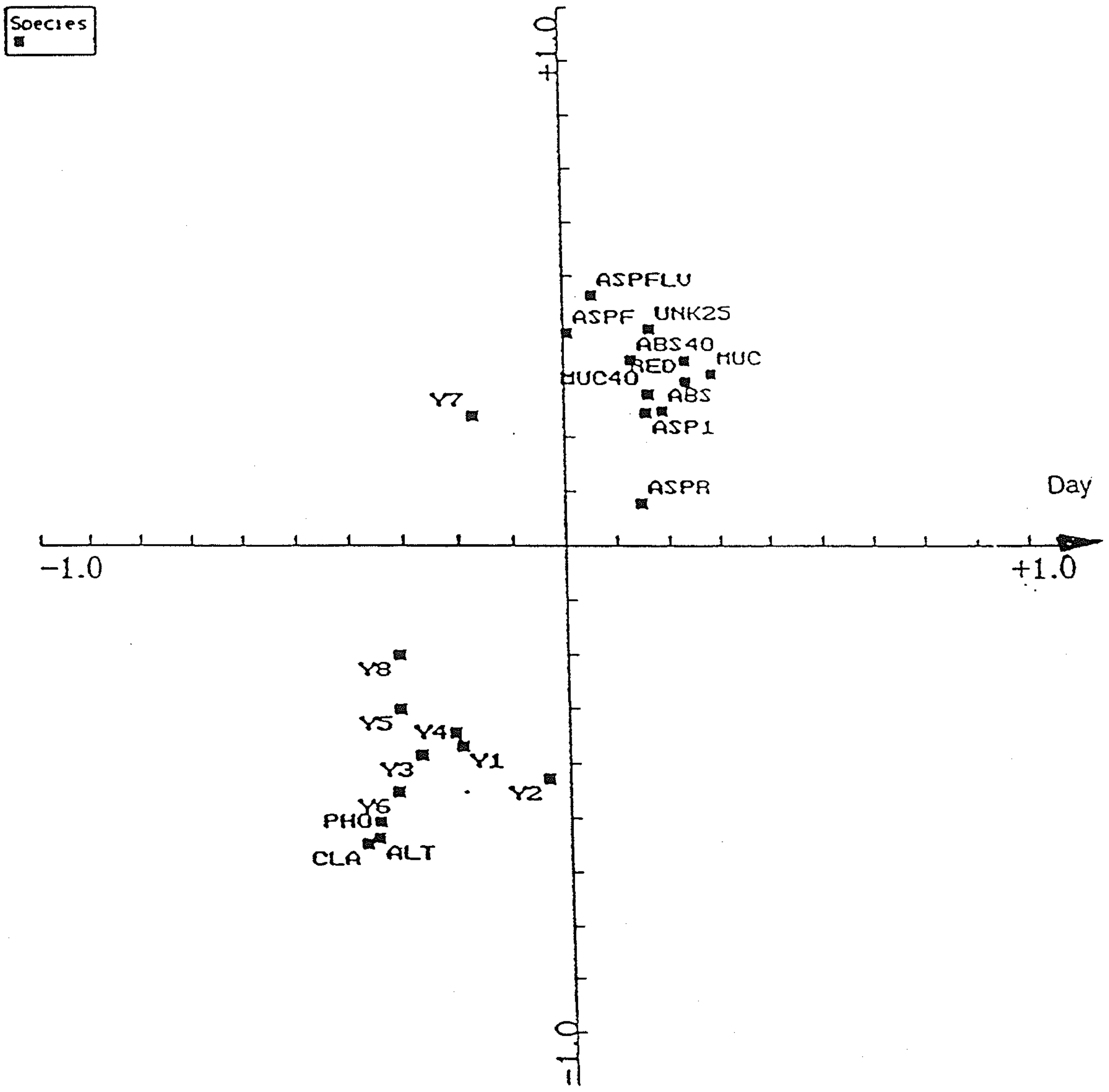

Soecies
+1.0
-1.0
-1.0
+1.0
Day
ASPFLU
ASPF
UNK25
ABS40
MUC
RED
MUC40
ABS
ASP1
Y7
ASPR
Y8
Y5
Y4
Y1
Y3
Y2
Y6
PHO
CLA
ALT

Figure 14. The CCA ordination diagrams for d 9 to 60 with sites (o), fungal species (●) and environmental variables (arrows). The sites and species are identified as in Fig. 11.

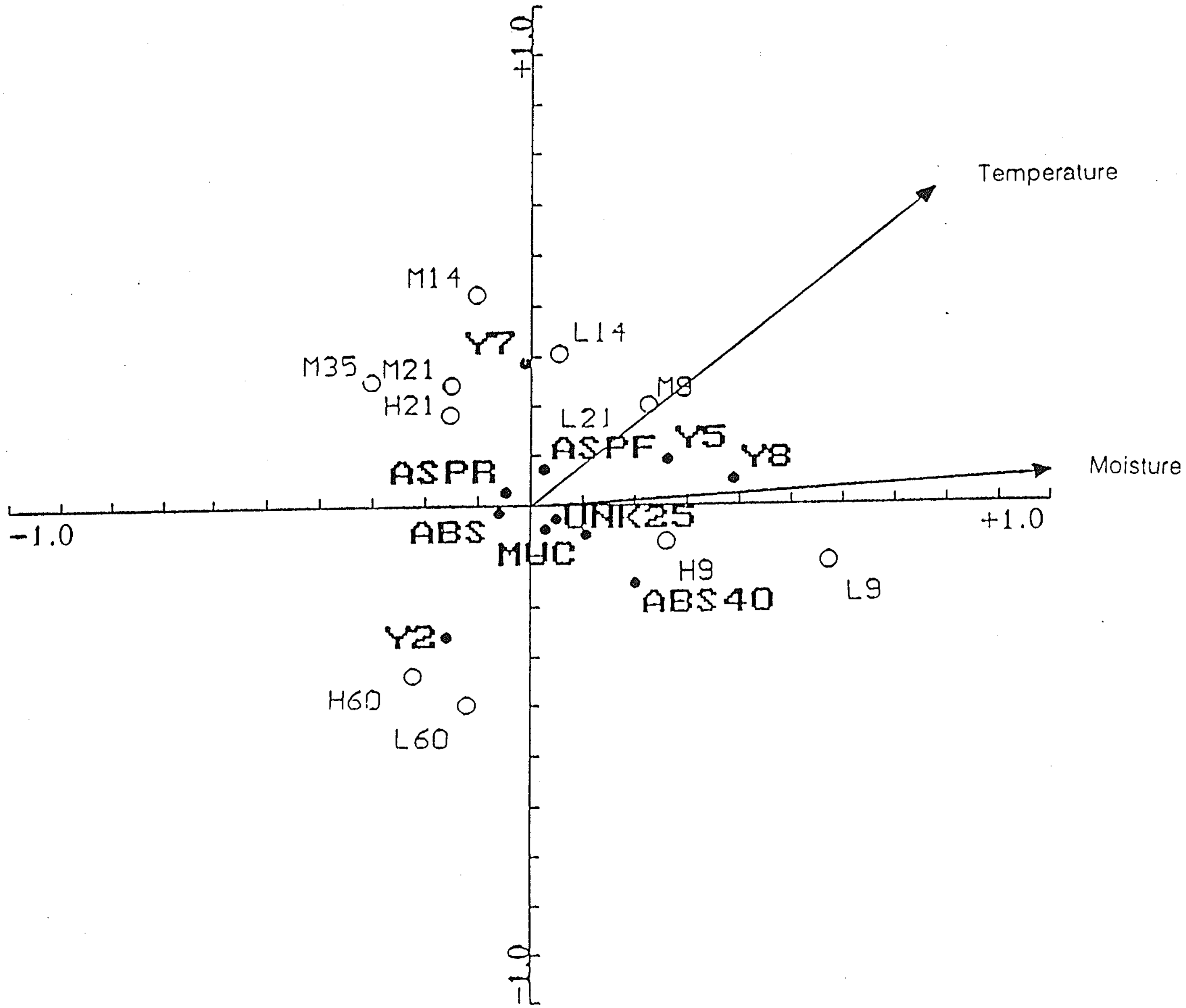

+1.0
-1.0
-1.0
+1.0
Temperature
Moisture
M14
Y7
L14
M35
M21
H21
M8
L21
Y5
ASPF
Y8
ASPR
ABS
UNK25
MUC
H9
L9
ABS40
Y2
H60
L60

TABLE 8. Fungal species in alfalfa forage baled and stored at three DM contents, Low (64.1-66.2%), Medium (71.9-73.2%) and High (75.4-77.4%)

	Dry matter content at baling, %																																						
	High													Medium													Low												
Days in storage	1	2	3	4	5	6	7	8	9	14	21	35	60	1	2	3	4	5	6	7	8	9	14	21	35	60	1	2	3	4	5	6	7	8	9	14	21	35	60
Phoma spp.	P	P	P	P	P		D							P		P	P	P		P							D	D											
Cladosporium	P		P												P												P	P	P										
Alternaria			P	P	P											P	P	P		P	P						P												
Yeast1	D	D	D	D	D	D	P	P	P	P		P		D	D	D	D	D	P	D	P	P			P	P		P			D	P							
Yeast2		P			P																				P														
Yeast3		P		P	P		P							P	P											P													
Yeast4		P			P	P	P	P	P	P		P		P	P	P	P	P	P	P		P				P	P												
Yeast5								P	P							P	P	P																					
Yeast6														P			P																						
Yeast7																	P																						
Asp1											P	P												P		P			P	P	D	P	P	P	P		P		
A.repens						P		D	D	D	D	D	D						D	P	D	D	D	D	D	D							P			P			
A. fumigatus																							P	P						D		P	P				P		P
Absidia spp											P		P												P								D	D	P		P		P
Absidia40																																	P				P		
Mucor spp																																				P			
Mucor40																							P	P		P							P	P					
Unident.													P																P										
Unknown25																																		P	P	D	P	D	D
Unknown40																																P		P	D	P	D	P	P

PSpecies that occurred in more than 50% of the bales in each treatment.

DSpecies used in calculation of dominance for each day and forage treatment.

Manuscript III: Determination of glucosamine as an estimate of fungal biomass in alfalfa hay by near infrared reflectance spectroscopy

ABSTRACT

The objective of this study was to verify the use of NIRS to measure glucosamine as an estimate of fungal biomass in alfalfa hay under diverse harvest and storage conditions. Sixty alfalfa hay samples collected from studies on hay storage over five growing seasons were used in the development of the calibration equation. Thirty samples were used to validate the calibration equation. A further 24 samples were used to test the calibration equation. The calibration coefficient of determination (R^2) was 0.84 with a standard error of calibration of 0.45 g kg^{-1} DM. The validation coefficient of determination (r^2) was 0.90, while the r^2 of test samples was 0.85. Standard errors of performance were 0.36 and 0.62 g kg^{-1}, respectively, for validation and test samples. Fifty-four bales which had been visually assessed for mold were used to establish a relationship between visually assessed mold and glucosamine. The correlation between glucosamine values and visual mold was low (r=0.40). It was concluded that NIRS could be used to quantify glucosamine in alfalfa hay and hence provides a rapid method for the estimation of fungal biomass in alfalfa hay.

INTRODUCTION

Making hay of good quality requires that the forage be dried to 80% DM for conventional square bales and 85% DM for large round bales (Jones and Harris 1980, Robertson 1983). These DM contents are considered to be safe for long term storage. However, such high DM contents can result in increased field losses; loss of DM in the field being mainly due to the disappearance of non-structural carbohydrates as a result of plant respiration and leaching by rain (Wilkinson 1981). Baling at low DM contents would result in low field losses and higher dry matter yields. However, forage stored at a low DM content has sufficient water activity to support fungal growth, resulting in molding and heating which increases dry matter losses in storage (Cherney et al. 1987; Rotz and Abrams 1988). Changes in composition during storage of low DM forage should be monitored and quantified to assess quality of the resulting hay and this step should include estimation of the amount of fungal biomass. Estimating the amount of fungal biomass in hay also becomes important in testing hay preservatives that are used to maintain hay quality. These preservatives are normally assessed by their relative ability to prevent fungal growth. Evaluation of animal performance when moldy hay is fed also requires the estimation of fungal biomass.

Two chemical methods currently used for estimation of fungal biomass involve the determination of either chitin (Donald and Mirocha 1977) or ergosterol (Seitz et al. 1979), since these entities are unique to the fungal cell wall. Estimation of fungal biomass using either one of these methods should give comparable results but the ergosterol assay has not been used in forages to date. Most commonly, fungal biomass in various agricultural products has been estimated by determining the chitin content of the products. Chitin is a constituent of the cell walls of most fungi and can be used as a measure of total (N-acetyl-D-glucosamine) fungal growth. Chitin, measured as its hydrolysis product glucosamine, has been used as an estimate of fungal biomass in such varied products as stored corn and soybean seed (Donald and Mirocha 1977), cereal grains (Rotter et al. 1989), and alfalfa hay (Roberts et al. 1987b; Wittenberg et al. 1989). Wittenberg et al. (1989) used an ion-exchange chromatography procedure to determine glucosamine in alfalfa hay. The advantage of the glucosamine assay as an estimate of fungal biomass is that it will reflect total mycelium and spores. The disadvantage is that it does not identify the fungi involved (Donald and Mirocha 1977). Other methods of estimating amount of fungal biomass such as plate and spore counts are time consuming and estimate viable mycelia only. Such methods are not suitable for a quick appraisal of fungal biomass contamination in hay. While the chemical methods discussed above are reliable and reproducible, they are expensive both in terms of the chemicals needed and time required to do the analysis.

Near infrared spectroscopy (NIRS) is a method which can reduce the time of analysis without sacrificing accuracy. The advantages of using NIRS include: speed of analysis, very low labor costs, simple sample preparation, nondestructive analysis of the sample, and pollution-free analysis (Williams 1987). The NIRS technique has been used in the analysis of crude protein, neutral detergent fibre (NDF), acid detergent fibre (ADF), and moisture in forages (Norris et al. 1976, Marten et al. 1983, Windham et al. 1987), in cereal grains and oilseeds (Williams 1975), and in wheat (Williams et al. 1983). Roberts et al. (1987b) used NIRS to estimate fungal biomass in alfalfa hay and concluded that it was a viable option to the chemical procedures. Near infrared spectroscopy also has been used to estimate fungal biomass in barley grain (Roberts et al. 1991).

The objective of this study was to verify the use of NIRS to measure glucosamine concentration in alfalfa hay derived from a wide range of harvest conditions. The relationship between visual mold and glucosamine also was investigated. To achieve variation, the sample set included a variety of alfalfa cultivars, 5 harvest seasons, 3 locations within Manitoba, and use of a range of additives and storage conditions.

MATERIALS AND METHODS

Ninety samples of alfalfa (*Medicago sativa* L.) hay containing varying amounts of fungal biomass determined as glucosamine were used. The samples covered a period of five growing seasons, from 1987 to 1992, and were from three sites within Manitoba. The cultivars used include Arrow, Beaver and common seed commonly grown in Manitoba. The hay was either from first, second or third cut. All hay was stored as small square bales, either under a tarp when outside or in a hay shed. Hay bales were core-sampled using a Penn State core sampler and at least four cores per bale were taken.

Some hay samples were obtained from a study using a chemical drying agent, potassium carbonate, applied at a rate of 40 ml kg^{-1} of forage DM. Thirty hay samples were derived from a series of trials evaluating bacterial additives. An additive containing viable *Lactobacillus plantarum* and *Pediococcus acidilactici* was applied to provide 1.8×10^5 and 1.6×10^5 total colony forming units (cfu) g^{-1} of forage DM, respectively, over two growing seasons in one trial. A second trial involved application of *Pediococcus pentosaceus* at a rate of 5×10^5 cfu g^{-1} of forage DM. Seventeen samples were obtained from trials testing two additives, one containing viable *Lactobacillus plantarum* and *Streptococcus faecium* applied at 3.6×10^5 cfu g^{-1} forage DM and the other, a *Lactobacillus* fermentation product applied at 0.09 - 0.11 ml g^{-1} forage DM. The remaining samples were obtained from untreated hay. Forage was baled and stored at three DM contents of 70-75%, 75-80% and 80-85% in all studies.

An extra 24 hay samples were used to test the calibration equation. Of these, 15 samples were obtained from Ontario using hay that was stored as round bales as opposed to the samples used in the calibration which were all from conventional square bales. The remaining 9 samples were from the previously described pool of samples.

A total of 54 samples from hay that had no visible mold or was moderately molded and which had been visually assessed for color, mold and dust by a rating system were used to establish a relationship between glucosamine content in hay and visual assessment for mold. Visual assessment was conducted using values ranging from 0 (no visual mold) to 5 (mycelial mat throughout the bale), with increments of 0.5 and at least two technicians graded each bale. The bales used in this study reflected high quality material typically associated with off-farm sales. The highest visual mold assessments were 1.5 which would reflect low amounts of visible mold.

Sample preparation was similar for all samples. Samples were freeze-dried and ground to pass a one mm screen in a Wiley mill. Fungal biomass estimates in ground samples were estimated by glucosamine analysis (Wittenberg et al. 1989).

Near infrared analysis.

The equipment used in this study was an NIRS scanning monochromator (Model 6500; NIRS Systems). NIR spectral analysis software was used in the development of the calibration equation and its subsequent validation.

Approximately 3-5 g of sample was placed in a sample holder and analyzed spectrally. Reflectance (log I/R) data were recorded between 500 and 1800 nm. Reflectance spectra were transformed using first derivative math transformation. The calibration equation was developed by regressing spectra of 60 samples against chemically determined glucosamine values. Details of the sample sets used in the study are shown in Table 9.

A sample of glucosamine-HCl (79%) was scanned to determine the main absorbance bands. The wavelengths chosen during calibration were compared to the spectrum. Similar spectral analysis was also performed on a sample of hay.

Stepwise multiple regression was used in the selection of appropriate wavelengths. The optimum calibration equation was chosen on the basis of a large calibration coefficient of determination (R^2), a small standard error of calibration (SEC), and high F values ($F \geq 10$) for each term selected in the regression (Westerhaus 1989). Optimum statistics for validation included a large validation coefficient of determination (r^2), a small standard error of performance (SEP), and small bias (Brown et al. 1987).

The R^2 resulted from regressing reflectance data against chemical data, and the r^2 resulted from regressing values predicted by the calibration equation against chemical data. The SEC was derived from the calibration set of samples and is the square root of the residual mean square error from the regression of laboratory values on NIR spectral data (Fairbrother and Brink 1990). The SEP was derived from the validation sample set and is the standard error of predicting laboratory values from NIR data.

RESULTS AND DISCUSSION

There were low correlations between laboratory glucosamine values and visual mold (r=0.40; Fig. 15), color (r=0.49), and dust (r=0.52). Hay color is associated with degree of heating during storage rather than degree of mold infestation. The amount of dust is associated with spore and mycelium load in hay. Wittenberg et al. (1989) reported a poor correlation between glucosamine and visible mold, which they attributed to accumulation of field mold. These data suggest that visual assessment should not be recommended as a method for hay mold determinations.

Four wavelengths (680, 1160, 1130, and 1490 nm) were required to produce the best equation for the analysis of glucosamine as an estimate of fungal biomass in alfalfa hay (Table 10). The wavelengths 1160 and 1490 nm occurred as peaks in the reflectance spectra of glucosamine-HCl and also in the reflectance spectra of moldy hay (Fig. 16) which offers at least some explanation as to why some of these wavelengths were chosen in the calibration equation. However, for multiterm equations, the wavelengths are so interdependent that interpretation of individual wavelengths is often difficult (Windham et al. 1988). The wavelength 680 nm lies in the visual range of the spectrum and can be used only in near infrared instruments that are capable of reading in this range. This wavelength can also be easily replaced by another before use.

The calibration coefficient of determination (R^2) was high and the SEC was low (Table 11). The R^2 was similar to that reported when glucose, arabinose, and pectin in forages were analyzed by NIRS (Fairbrother and Brink 1990). The R^2 and SEC also were similar to those reported when hay was analyzed spectrally for fungal biomass (Roberts et al.1987b) but the SEP in this current study was lower, possibly due to the wider range of harvest conditions associated with samples used in the current study.

Validation of the calibration equation is shown in Table 11. The validation coefficient of determination (r^2) was high and compares to that reported by Roberts et al. (1987b). The mean difference (bias) between laboratory values and NIRS determined values was -0.17 g kg^{-1} DM and the slope and intercept were 0.93 and 0.35, respectively. Testing the calibration equation using a set of test samples showed statistics which were different from the initial validation. For test samples, the r^2 was lower and SEP and bias higher than those for validation.

Differences in slope between the validation set and test samples can, to a certain extent, be attributed to differences in the ranges of glucosamine concentrations. The lower r^2 and higher SEP and bias for test samples can be explained by location differences and also differences in storage conditions. Most of the test samples came from hay that was baled in large round bales. These test samples were not included in the calibration and this would affect the validation statistics as is clearly shown in this case. Ideally some of these test samples should be included in the calibration sample set. Therefore, before this equation can be utilized on a practical basis, it will require the addition of more samples to incorporate all sources of variance including such aspects as differences in location, sample handling, and composition (Williams 1987).

CONCLUSION

Visual appraisal, which is currently practised in hay marketing, is limited in its use because hay may appear to have no visible molding but still have large amounts of fungal biomass. Near infrared reflectance spectroscopy can be used to analyze for glucosamine for estimation of fungal biomass in alfalfa hay and glucosamine can be quantified by this method using a wide variety of wavelengths. Which wavelengths to adopt would be determined by instrumentation capability and availability. The method would also be useful in situations where a rapid appraisal of the fungal biomass status of hay in marketing situations is required.

However, the calibration equation should be updated as samples resulting from new circumstances such as year, harvest method or storage conditions are encountered.

Figure 15. Relationship between glucosamine as determined by ion-exchange chromatogrphy and visual mold scored as 1=no visible mold to 5=visible mycelium throughout the bale with increments of 0.5

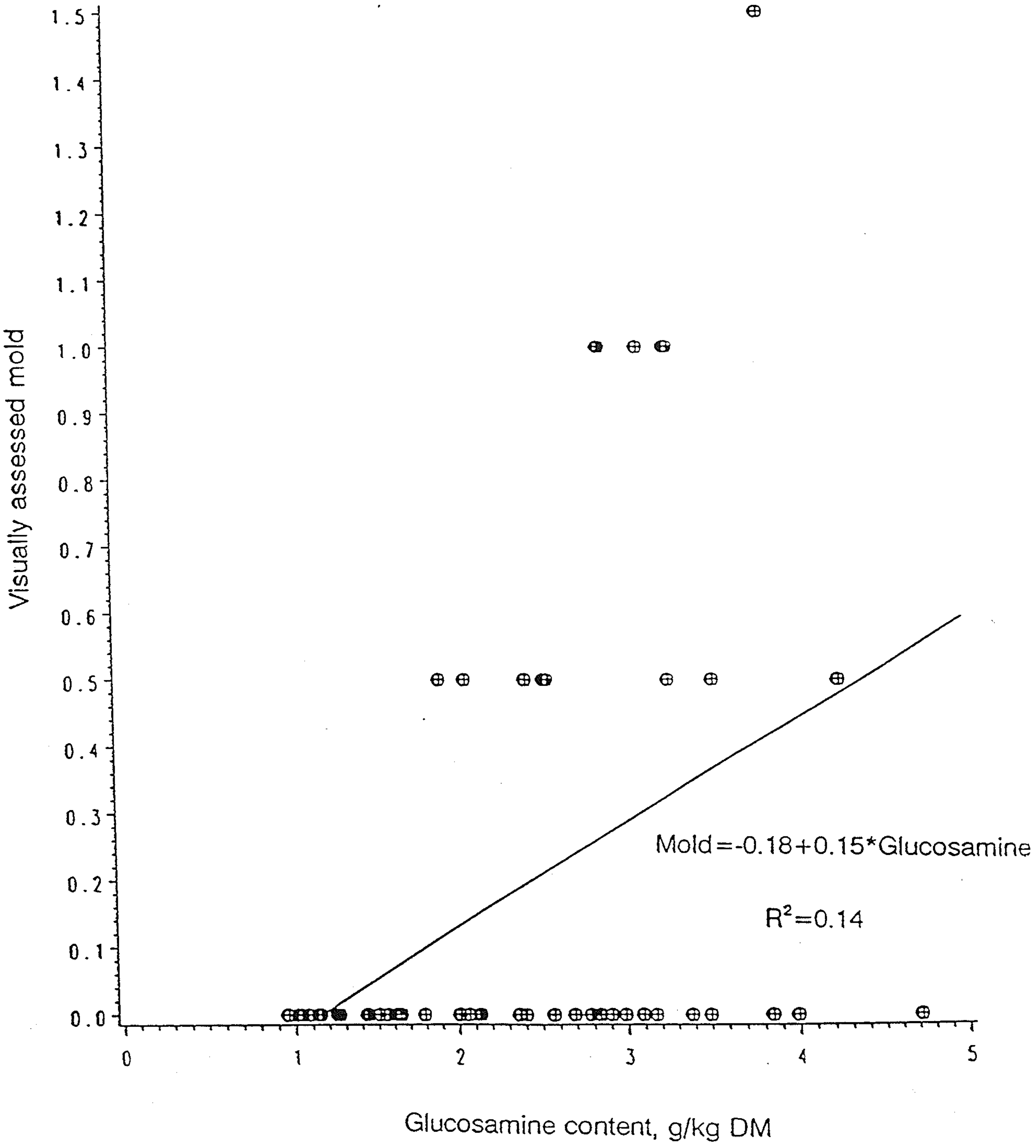

1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Visually assessed mold
0
1
2
3
4
5
Glucosamine content, g/kg DM
Mold=-0.18+0.15*Glucosamine
R²=0.14

Figure 16. Near infrared spectra of glucosamine-HCl (—) and a moldy alfalfa hay sample (-----). Wavelengths 1160 nm (A) and 1490 nm (B) occurred in the calibration equation.

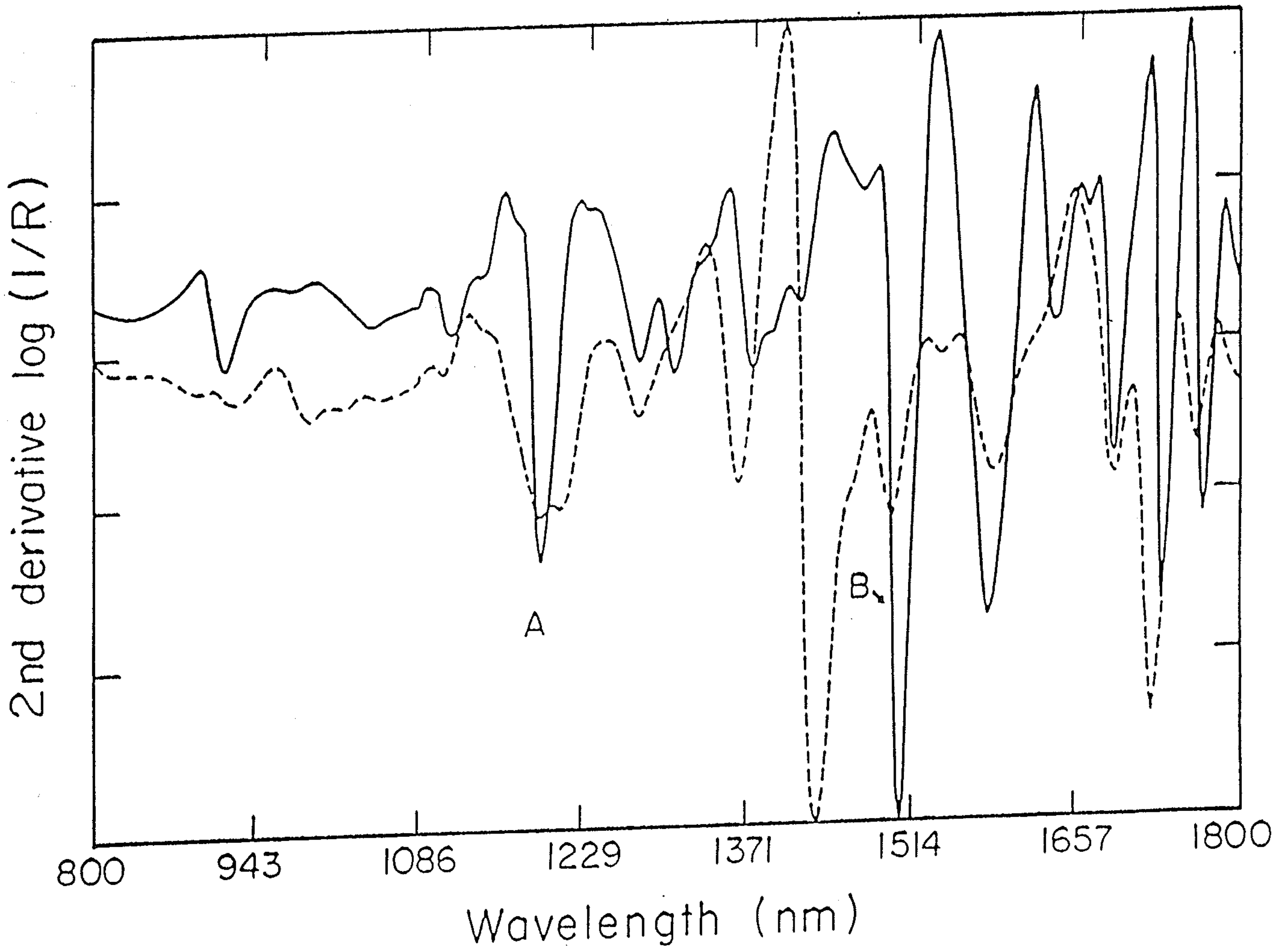

2nd derivative log (1/R)
A
B
800
943
1086
1229
1371
1514
1657
1800
Wavelength (nm)

TABLE 9. Characteristics of the calibration, validation and test sample sets.

	Calibration	Validation	Test
n	60	30	24
Mean, g kg^{-1}	3.07	2.95	3.80
S.D., g kg^{-1}	1.11	1.07	1.55
High, g kg^{-1}	5.60	4.96	8.25
Low, g kg^{-1}	1.03	0.98	1.72

TABLE 10. Wavelengths, coefficients, and F values utilized in the analysis of glucosamine as an estimate of fungal biomass in alfalfa hay

Wavelengths (nm)	Coefficients	F
-	15.15	-
680	77.85	133.04
1160	-2756.71	57.50
1490	866.89	10.32
1130	3098.11	41.17

TABLE 11. Calibration and validation statistics for NIRS analysis of glucosamine as an estimate of fungal biomass in alfalfa hay

	n	$R^{2,y}$	$r^{2,z}$	mean	SE	Bias	Slope
				---------g kg^1 DM ---------			
Calibration	60	0.84	-	3.07	0.45	-	-
Validation	30	-	0.90	2.95	0.36	-0.17	0.95
Test samples	24	-	0.85	3.80	0.62	0.51	0.96

[y] Calibration coefficient of determination.

[z] Validation coefficient of determination.

Manuscript IV: Effect of fungal biomass in molded alfalfa hay on preference by dairy calves with no previous exposure to moldy feeds.

ABSTRACT

A study to evaluate preference of alfalfa hay containing different amounts of fungal biomass, as estimated by the glucosamine assay, was carried out with dairy calves. Alfalfa forage harvested at different maturities and stored at varying dry matter contents was chopped and blended to produce four forage treatments. The four treatments included: hay that had low neutral detergent fibre content (NDF) and low amount of fungal biomass (LL), high NDF and low fungal biomass hay (HL); high NDF and moderate fungal biomass hay (HM); and, high NDF and high fungal biomass hay (HH). Forage treatments were offered in pair combinations with two feeders for each calf, treatments being switched from one feeder to the other on alternate days. The four forage treatments were offered in such a way that all 6 pair combinations were tested in each period. Two trials were performed with calves, each trial designed as a 6x6 latin square with preference data collected for six day periods and analyzed as a split plot. Difference in intake of pairwise offerings of forage treatments was the response variable. Hay preference declined as either fibre content of hay ($P<0.05$) or amount of fungal biomass in hay increased ($P<0.05$). Forage intake of each treatment relative to mean intake was 3.3, 0.8, -0.5 and -3.7 kg/6d for LL, HL, HM and HH treatments, respectively. Low preference for molded hay would probably result in greater feed sorting and lower intakes where animals have a choice of feedstuffs.

INTRODUCTION

Baling and storing forage at low DM content reduces DM losses associated with prolonged exposure in the field. However, low DM storage subjects forage to further losses that are associated with increased microbial activity during storage (Nehrir et al. 1978; Breton and Zwaenepoel 1991). Soluble nutrients such as non-structural carbohydrates are lost during low DM forage storage but the concentration of cell wall, bound nitrogen and lignin increases (Miller et al. 1967; Cherney et al. 1987; Rotz and Abrams 1988). The accumulation of fungal biomass also contributes to this loss of hay quality. However, the relationship between amount of fungal biomass in hay relative to extent of quality decline is poorly defined.

Mertens (1994) defined preference as the relative acceptability of a feed when given the choice among two or more feeds that are available in a cafeteria-type feeding situation. Palatability affects the preference of a feed when several feeds are offered and, since preference can be readily determined, palatability can be estimated from such a measurement.

In many cattle operations hay is offered as a separate ingredient in rations. Low palatability may result in greater feed sorting and lower intakes where animals are offered a choice of feedstuffs. The purpose of this study was to determine whether fungal biomass in molded alfalfa hay affects preference, and hence, palatability, for hay fed to calves with no previous exposure to molded feedstuffs.

MATERIALS AND METHODS

Alfalfa (*Medicago sativa* L.) forage harvested at different maturities, baled and stored at varying DM contents for a minimum of 60 d were core-sampled and analyzed for NDF and glucosamine. Rations were formulated using these hays to generate four treatments (Table 12): hay that had low NDF content and a low amount of fungal biomass (LL). The other three forage treatments were blended to contain about 49% NDF, DM basis, with increasing amounts of fungal biomass as determined by the glucosamine assay, and were designated HL, HM, and HH, for low, medium, and high amounts of fungal biomass, respectively. Forages were blended at the time of chopping (New Holland forage harvester) to ensure uniformity in composition. The HL treatment contained 10% barley straw to achieve same NDF content as the HM and HH treatments. The crude protein content of the forage treatments was targeted to be 18%, DM basis.

Forage treatments were offered to two groups (n=6) of holstein calves in two experiments. Calves were approximately 5 and 3.5 months of age and weighed 150.2±20.9 kg and 132.4±15.7 kg, in experiments A and B, respectively. Care was taken to ensure minimal prior exposure to mold in feed for calves used on test. Calves were housed in individual pens equipped with two feeders and automatic waterer located on the same side of the pen. In each period of six days, two forage treatments were offered to each calf in such a way that all pair combinations of forage treatments were fed per period. Equal amounts of forage treatments were offered during each feeding. Each calf was offered all six pair combinations of forage treatments once in each experiment. Forage treatments were alternated between the two feeders daily in each period to avoid bias due to feeder preference. Care was taken to ensure that each feeder had hay at all times so as not to remove the element of choice.

Calves also were offered a grower ration (Table 13) every day at 8:00 AM, at one third of *ad libitum* intake, based on DM intake records one week before the beginning of the study. The grower ration was removed at 10:00 AM, feeders were cleaned, and the forage treatments were offered for the rest of the day. Weighbacks were removed, weighed and sampled daily prior to feeding the grower ration. Forage and grower ration samples were collected every day by grab sampling. Forage samples were composited by treatment, subsampled for microbiological analysis and the remainder freeze-dried for DM determination and ground to pass a 2 mm sieve in a Wiley mill. Samples were analyzed for crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), cellulose, hemicellulose, lignin, acid detergent insoluble nitrogen, water-soluble carbohydrate and glucosamine. Dry matter determinations of weighbacks were done using a forced-air oven set at 60°C for 48 hours. The grower ration was composited, dried, ground and analyzed as for forage samples with the addition of calcium and phosphorus analyses.

Calves in this study were cared for in accordance with guidelines established by the Canadian Council of Animal Care.

Chemical analysis

Forage and grower ration samples were analyzed for N content by the Micro-Kjeldahl method (Association of Official Analytical Chemists (Method 47.023, AOAC 1984) using a Tecator 1030 analyzer (Tecator, Hoganas, Sweden). Neutral detergent fibre was determined by the procedure of Goering and Van Soest (1970) modified to exclude decalin and sodium sulphite. Acid detergent fibre was determined by Method 7.076 (AOAC (1984). Both these analyses were carried out on a Tecator 1020 Fibertec system (Tecator, Hoganas, Sweden). Acid detergent insoluble nitrogen was performed on ADF residues using the Micro-Kjeldahl procedure. Cellulose and lignin content in forage samples were determined by sequential analysis (Robertson and Van Soest 1981). Hemicellulose was obtained as the difference between NDF and ADF (Robertson and Van Soest 1981). Estimation of fungal biomass in forage treatments was done by the glucosamine procedure (Wittenberg et al. 1989). Water-soluble carbohydrate content in forage treatments was determined following extraction in 80% (v/v) ethanol using the procedure described by Jermyn (1975). Calcium and phosphorus in the calf grower ration were determined following dry ashing using Method 7.101 of the AOAC (1984). Calcium was determined by atomic absorption spectroscopy (Method 7.096) and phosphorus was determined colorimetrically (Method 7.125, AOAC 1984).

Forage samples also were screened for mycotoxins, namely, aflatoxins, ochratoxins, citrinin, and zearalenone, using thin layer chromatography as described by Scott et al. (1970). Trichothecene mycotoxins were screened for by the procedure of Trucksess et al. (1987). For verification of mycotoxin analysis, a subsample (HH) was sent to the Veterinary Diagnostics Laboratory (North Dakota State University, Fargo ND).

Microbiological analysis

Approximately 15 ml of a sterile wash solution (0.1% Tween 80 solution) was added to a 2 g subsample of hay in sterile screwcap test tubes. Samples were then shaken at 400 rpm for 10 min. The wash solution was removed and samples placed in sterile centrifuge tubes which were centrifuged at 7000 xg for 20 min. After centrifugation the supernatant was aspirated off and 2 ml of sterile wash solution was added into each centrifuge tube to resuspend the pellet. Samples were then serially diluted using 0.1% Tween 80 solution. The dilutions were plated on Dichloran Rose Bengal agar (King et al. 1979) and plates were incubated at either 25°C for 5 days before counting and identification of fungal species. Identification was based on morphological characteristics (Pitt and Hocking 1985). Isolates were sent to the International Mycological Institute (Biosystematics Services, Bakeham Lane, Surrey, UK) for verification. Counts were corrected for DM by drying the remaining subsample in a forced-air oven at 60°C for 48 hours.

Statistical analysis

The preference study was carried out with two groups of six calves using a design originally described by Dayton and Morril (1974) for preference studies.

Data were analyzed as a repeated latin square design in a split plot, where forage treatments in each pair-combination are subplot treatments within animal, period and pair whole-plots (Kemp et al. 1990). Data analysis was carried out using General Linear Models of the Statistical Analysis System (SAS 1985). Differences in intake between forage pair combinations were tested using single degree of freedom contrasts, following a significant F test. One calf contracted pneumonia in one period of experiment B and interim data were not included in the analysis.

RESULTS AND DISCUSSION

The nutrient profiles of HL, HM and HH treatments were comparable although actual NDF concentration for HH was higher than that for which this treatment was formulated (Table 12). Also, soluble carbohydrate concentrations for the HL and LL were higher than for the other two treatments. The nutrient profile of the LL treatment was different from the other treatments in having lower concentrations of NDF, ADF and ADIN. The comparison of two hays having different NDF contents allowed us to verify that the experimental design was sensitive. Forage treatments HM and HH were observed to be extremely dusty during chopping and hay used to produce these two forage treatments generally had visible mold in 50 to 100% of the bale.

Unidentified thermotolerant hyphomycetes made up a major proportion of counts in both HM and HH forage treatments (Fig. 17). Attempts to have this species identified were not successful and only resulted in placing it into a class, hyphomycetes. Common storage fungi which belong to this class include *A.*

fumigatus, *Humicola lanuginosa*, *Paecilomyces variotti*, and *Penicillium cyclopium* among others (Breton and Zwaenepoel 1991). Counts of *Aspergillus glaucus* also were high in HM and HH treatments. Forages HM and HH also were characterized by the presence of *A. versicolor* and *A. fumigatus,* which were not detected in LL and HL treatments.

The presence of field fungi, *Cladosporium* spp., *Alternaria* spp. and *Phoma* spp. in LL and HL treatments is indicative of forage which was not exposed to extensive heating during storage. The HM and HH forage treatments had a higher proportion of fungal species typically found in hay which has undergone extensive heating during storage (Kaspersson et al. 1984; Lacey 1989; Breton and Zwaenepoel 1991). The presence of species such as *A. fumigatus* and *A. versicolor* in hay is an indicator of extensive damage of hay during storage (Lacey 1989).

The forage treatments were negative in mycotoxins for which they were screened. Screening for mycotoxins was important to ensure that the presence of mycotoxins was excluded as being responsible for any observed effects in preference. Mycotoxins have not been isolated from moldy hay even when species of toxin-producing fungi were present in the hay (Lacey 1975). However, with increasing use of forage preservatives, there is a trend to store forage at lower DM contents which increases greatly the potential for mycotoxin production. Conditions that could lead to mycotoxin production in hay are poorly understood, and it seems that the problem when forage is stored at low DM contents is that of fungal biomass accumulation and utilization of nutrients in forage rather than known secondary metabolites.

The most preferred hay was low in both NDF content and fungal biomass resulting in higher intake in all pair combinations in which it was offered (Table 14). The least preferred treatment was that with the highest amount of fungal biomass. For treatments which had similar NDF levels, that is, HL, HM, and HH, preference decreased ($P<0.05$) as the amount of fungal biomass in hay increased (Table 14). The LL forage treatment was preferred over the HL forage treatment, suggesting that the difference in preference between these two treatments was due to the high NDF content of the HL treatment. The order of preference of forage treatments was LL > HL ≥ HM > HH , based on intake of each forage in pair combinations (Table 15).

Studies with fibrous feeds have shown a relationship between intake of a feed and its cell wall content. The intake of a fibrous feed declines with increasing cell wall content of that feed (Van Soest 1982). In studies of moldy hay, dry matter intake is generally not influenced when animals are fed moldy hay as the sole or major dietary feedstuff (Smith et al. 1989; Wittenberg and Moshtaghi-Nia 1990). However, direct comparisons between results of intake studies and those of a preference study cannot be made. Intake studies evaluate only a single feed at a time and, as a result, choice and selection, are eliminated (Van Soest 1982).

The low fungal biomass treatments, LL and HL, had a green color which is associated with good quality hay. Forage treatments HM and HH had portions in the bale that were dark brown to grey in color, an indication that the hay had undergone heating in storage. Whether hay color was important in preference differences among forage treatments is questionable since studies have shown no change in eating patterns of sheep or steers that were offered feeds altered by addition of artificial colors (Church 1976). Besides, hay color by itself is not necessarily a good indicator of hay quality. Hay may look green but still contain fungal biomass especially when the dominant fungal species in that hay are members of the *A. glaucus* group (Lacey 1975).

CONCLUSION

Hay is frequently offered as a separate feed to cattle and therefore preference can be an important factor in feed utilization. Animals will avoid consuming moldy hay when given a choice of feeds. This study shows that preference can be affected specifically by fungal biomass contamination in molded hay and that preference declines as amount of fungal biomass increases. It is concluded that fungal invasion during hay harvest and storage can reduce preference, and hence palatability mostly through fungal biomass contamination.

Figure 17. Predominant fungal species and counts in forage treatments having different amounts of fungal biomass.

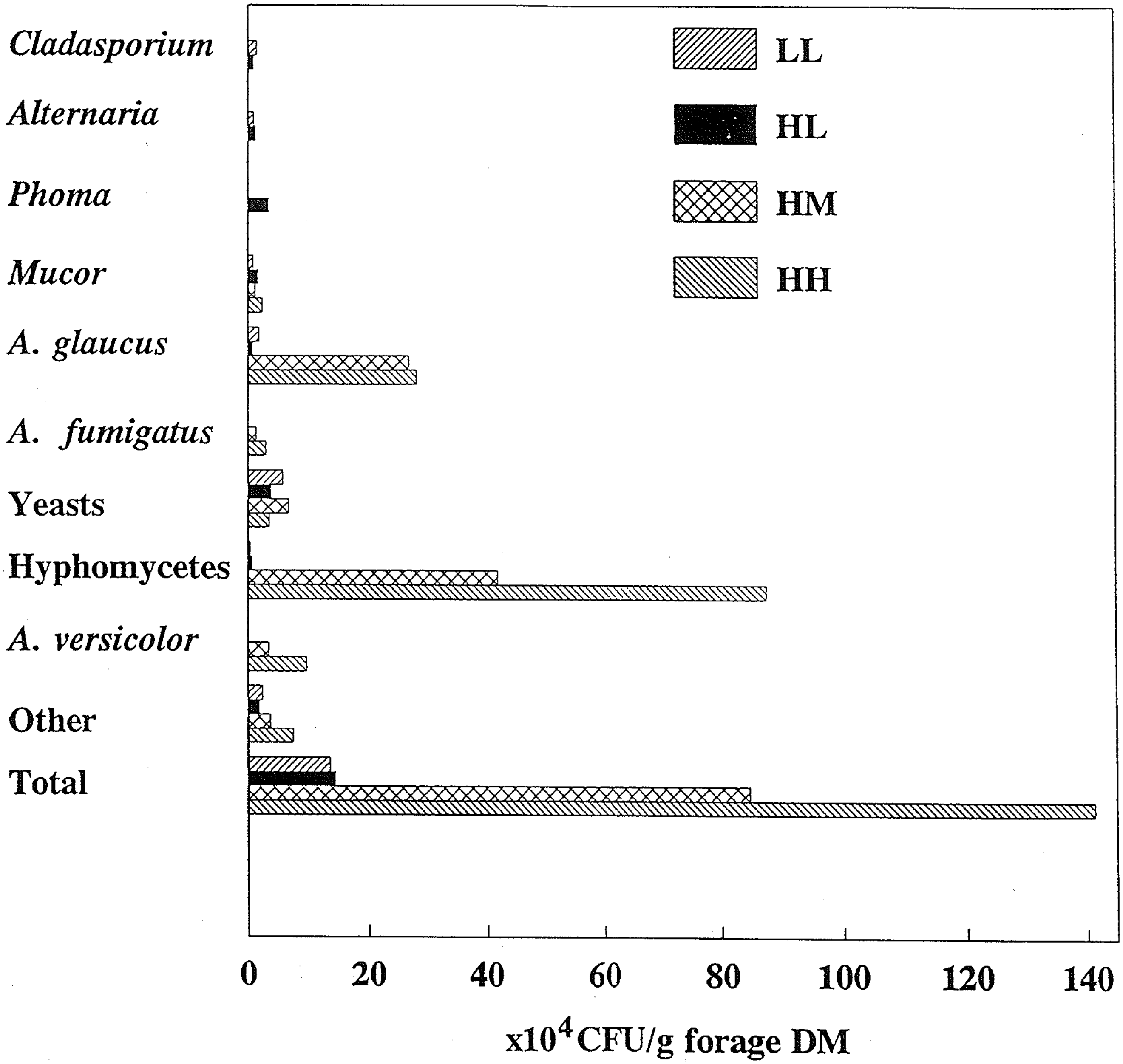

Cladasporium
Alternaria
Phoma
Mucor
A. glaucus
A. fumigatus
Yeasts
Hyphomycetes
A. versicolor
Other
Total
LL
HL
HM
HH
0
20
40
60
80
100
120
140
x10^4 CFU/g forage DM

TABLE 12. Chemical composition of the forage treatments offered to calves in the preference study.

	Forage treatment			
	LL[X]	HL	HM	HH
Dry matter, %	85.1	87.4	86.8	85.5
Crude protein, % DM	18.4	17.6	18.6	19.6
Neutral detergent fibre, %DM	43.7	49.1	48.2	52.8
Acid detergent fibre, %DM	33.2	36.1	37.0	38.2
Acid detergent insoluble N, %total N	5.6	7.1	7.7	8.9
Cellulose, %DM	20.2	23.6	24.1	25.5
Hemicellulose, %DM	10.5	13.0	11.1	14.6
Lignin, %DM	8.5	9.3	10.0	10.9
Soluble carbohydrate, %DM	5.3	5.9	2.7	2.6
Mycotoxins[z]	ND[Y]	ND	ND	ND
Glucosamine, g kg^{-1} DM	1.5	1.7	3.2	4.3

[X] LL=Low NDF content and low fungal biomass forage, HL=high NDF and low fungal biomass, HM=high NDF and medium fungal biomass, HH=high NDF and high fungal biomass content.

[Y] Not detected.

[z] Common mycotoxins screened for include: Deoxynivalenol, fusarenon-X, nivalenol, aflatoxins, ochratoxin A, zearalenone, citrinin, patulin, sterigmatocystin, and penicillic acid.

TABLE 13. Ingredients and chemical composition of the concentrate ration fed to calves in the preference study.

Ingredient, % as fed	
Rolled barley	73.2
Canola meal	14.0
Distiller grains	6.0
Beet molasses	4.0
Mineral premix^{W}	0.8
Vitamin premix^{X}	0.6
Biophos^{Y}	0.8
Limestone	0.4
Selenium mix^{Z} (200 mg kg^{-1})	0.2
Chemical composition, % as fed	
Crude protein	15.4
Neutral detergent fibre	30.2
Acid detergent fibre	9.3
Acid detergent insoluble N, %Total N	8.7
Calcium	0.4
Phosphorus	0.5

^{W}Contained (mg kg^{-1}): Potassium iodide 0.6, Copper sulphate 15, zinc oxide 60.5, manganese oxide 61.2, magnessium oxide 0.65 gkg^{-1}, and cobalt-iodized salt 6.6 g/kg.

^{X}Formulated to provide (IU kg^{-1}): vitamin A 15,000, Vit. D_3 3,000, and vitamin E 90.

^{Y}Contained 21.0% P and 18.0% Ca.

^{Z}Contained (%): Sodium selenite 0.045, mineral oil 1.5, rice hulls 40, and limestone 58.46.

TABLE 14. Pairwise differences in intake (kg/6 d) by calves fed forage treatments with different amounts of fungal biomass.

Treatment Pairing[z]	Difference	SE	Significance
LL vs HL	2.5	0.6	$P<0.001$
LL vs HM	3.8	0.6	$P<0.001$
LL vs HH	7.1	0.6	$P<0.001$
HM vs HH	3.2	0.6	$P<0.001$
HL vs HM	1.3	0.6	$P<0.05$
HL vs HH	4.5	0.6	$P<0.001$

[z] LL=low NDF content and low amount of fungal biomass; HL= high NDF, low fungal biomass; HM= high NDF and medium fungal biomass, and HH = high NDF and high fungal biomass content.

TABLE 15. Dry matter intake (kg/6 d) by calves of forage treatments with different amounts of fungal biomass.

Forage treatment	Intake (kg)	SE	Rel. to Mean[X]
LL[Y]	10.6a	0.4	3.3
HL	8.1b	0.4	0.8
HM	6.8b	0.4	-0.5
HH	3.6c	0.4	-3.7

[X] Values are either increases or decreases in intake relative to the overall mean (7.3 kg).

[Y] LL=low NDF, low fungal biomass hay; HL=high NDF, low fungal biomass hay; HM=high NDF, medium fungal biomass hay, and HH=high NDF, high fungal biomass hay.

a-cValues within the same column with a different letter differ ($P<0.05$).

Manuscript V: *In situ* digestion kinetics of feedstuff dry matter, crude protein and neutral detergent fibre as influenced by fungal biomass in molded alfalfa hay

ABSTRACT

The objective of this study was to establish whether fungal biomass typically observed in molded hay would affect digestion kinetics of feedstuffs in the rumen. Feeds assessed included corn grain, barley grain, canola meal, alfalfa hay, and barley straw. Three alfalfa hay treatments with similar nutrient profiles but varying in fungal biomass content were fed to late lactation, ruminally cannulated cows in two 3x3 latin squares. The hay treatments contained 1.6, 2.5 and 3.3 g glucosamine kg^{-1} DM. Mycotoxins were not detected in the hay treatments. A concentrate ration also was offered to the cows such that the hay to concentrate ratio was 70:30, DM basis. Test feeds were incubated in the rumen for 0.1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h. Hay intake was similar across treatments ($P>0.05$) and averaged 2.1% BW. Volatile fatty acids and rumen pH were not affected ($P>0.05$) by fungal biomass in alfalfa hay. Rate of DM degradation and effective DM degradability of barley straw was higher ($P<0.05$) when incubated in the rumen of cows fed hay with higher amounts of fungal biomass. Fungal biomass in hay did not affect ($P>0.05$) rate and extent of DM degradation in corn grain, barley grain, canola meal and alfalfa hay; rate and extent of CP degradation in canola meal and alfalfa hay, or rate and extent of NDF degradation in alfalfa hay and barley straw. Presence of fungal biomass in alfalfa hay does not affect the rate and extent of DM, CP and NDF degradation of feedstuffs commonly used in ruminant rations but may affect rate and extent of DM degradation of highly lignified feedstuffs such as barley straw.

INTRODUCTION

Harvest and storage conditions can affect hay quality and quantity. Buckmaster et al. (1990) estimated that total dry matter (DM) losses during harvest, storage and feeding of alfalfa hay were nearly 25%. These losses can be reduced substantially by reducing field losses due to respiration, rain and mechanical losses, and this can be achieved by baling forage at DM contents lower than 80-85%. However, such forage is likely to undergo molding with an accompanying reduction in hay quality.

Studies have shown that, in general, DM intake is not different between moldy and nonmoldy hay while digestibility of nutrients such as DM, CP, and energy is lower in moldy hay (Smith et al. 1989; Wittenberg and Moshtaghi-Nia 1990). However, general comparisons of studies on nutritive value of moldy hay are difficult due, in part, to no or poor estimation of fungal biomass contamination and to differences in nutrient profiles between moldy and nonmoldy hays being compared. As such, effects of either different nutrient profiles of hay or those of fungal biomass in hay have to be clearly differentiated to allow specific evaluation of one or the other.

Dried forage can comprise from 0 to 100% of total diet DM in dairy cattle rations. As well as establishing change in nutritive value of the moldy hay, research also is needed to evaluate how digestion of other feeds in dairy cattle diets are affected when they are fed in combination with molded hay. The objective of this study was to establish whether fungal biomass typically observed in molded alfalfa hay would affect digestion kinetics of feedstuffs in the rumen. Corn grain, barley grain, canola meal, alfalfa hay, and barley straw were evaluated in cows fed alfalfa hay with different amounts of fungal biomass, using the *in situ* technique.

MATERIALS AND METHODS

Alfalfa (*Medicago sativa* L.) forage harvested at varying maturities, and stored at varying DM contents was used to prepare the forage treatments that were fed to the cows. Core samples from the hay lots were analyzed for NDF, ADF, CP, and glucosamine to formulate three hay treatments having similar nutrient profiles but differing in the amount of fungal biomass. Hay from representative lots was blended at the time of chopping (New Holland) to ensure uniformity of composition. Forage treatments designated as low (LFB), medium (MFB) and high(HFB) fungal biomass contained 1.6, 2.5 and 3.3 g glucosamine kg^{-1} DM, respectively (Table 16). Ten percent (w/w) barley straw was added to the LFB treatment to equalize fibre content of the forage treatments.

Forage treatments were offered *ad libitum* to three late lactation cows (719±61.5 kg) that were fitted with a large diameter rumen cannula. Cows were housed in individual pens during the adaptation period and were moved to tie-stalls in the last week of each period. Forage treatments were offered twice daily, at 9:00 AM and 4:00 PM. A concentrate ration (Table 17) was also offered daily and was dispensed at 2 h intervals using automated feeders (Ankom, Fairport, NY) to stabilize the rumen environment. The forage to concentrate ratio achieved was

70:30, DM basis. Forage weighbacks were collected every morning before the AM feeding, weighed, and subsampled for DM determination by oven-drying at 60°C for 48 h.

Each forage treatment was fed to cows for 21 days in each period, the first 14 days of which were for adaptation. Forage and concentrate samples were collected daily in the last 7 days of each period. Forage samples were composited by treatment with one subsample used for DM determinations by freeze-drying and a second subsample held for microbiological analysis. Dried forage samples where ground to pass a 2 mm screen in a Wiley mill before analysis for CP, NDF, ADF, ADIN, total soluble carbohydrate, cellulose, hemicellulose, lignin, and glucosamine. Rumen fluid samples were collected on day 15 of each period, 2 h after AM feeding. Rumen fluid pH measurements were taken, using a pH meter, immediately following fluid collection after which samples were then frozen for subsequent volatile fatty acid (VFA) analysis.

Cows in this study were cared for in accordance with guidelines established by the Canadian Council of Animal Care.

In situ technique

Barley grain, canola meal, and corn grain were ground to pass a 2 mm screen, and alfalfa hay and barley straw were ground to pass a 5 mm screen (Wiley mill) before being placed into multi-filament, low N, polyester fabric bags with a porosity of 53±10 μm (Ankom, Fairport, NY). Approximately 0.8 g of barley grain, canola meal, and corn grain were placed into small (4 x 5 cm) heat-sealed bags. Approximately 1.5 g of alfalfa hay and barley straw were each put into bigger bags (6 x 10 cm). Sample size to bag surface ratios were 20 mg cm $^{-2}$ for barley grain, canola meal, and corn, and 12.5 mg cm $^{-2}$ for alfalfa hay and barley straw. These ratios fall within recommended guidelines for concentrates and forages to achieve optimum bacterial attachment (Nocek 1988; Michalet-Doreau and Ould-Bah 1992).

Duplicate bags of each test feed were incubated in the rumen for 0.1, 2, 4, 6, 8, 12, 24, 48, and 72 h for barley grain, canola meal, and corn. In addition to the above incubation times, alfalfa hay and barley straw were also incubated for 96 h. Empty sealed bags were included at each incubation time to serve as blanks to correct for attachment of rumen contents, feed particles and microorganisms to the bags. The bags were introduced into the rumen at different times but were removed all at once in a reverse sequence (Nocek 1988).

Following removal from the rumen, the bags were washed immediately with cold water in a wringer-type washing machine for 10 min, the water drained, and the process repeated for 5 min. Bags were then dried in a forced-air oven at 60°C for 48 h for DM determination. The duplicate bags containing canola meal, one bag per duplicate containing alfalfa hay, and the blank bags were analyzed for N content. The second bag of each duplicate of alfalfa hay and all bags containing barley straw were analyzed for NDF. The bags containing alfalfa hay and barley straw were opened and the samples were reground to pass a 2 mm screen before NDF analysis.

Calculations.

Percent disappearance of DM, CP, and NDF for the respective feeds in bags was calculated as 100 minus the remaining residue, expressed as %, at each rumen incubation time. Blank bags were used to correct for DM and N disappearrance by using change in weight and N content for correction, respectively. The disappearance parameters for DM, CP and NDF for barley grain, canola meal, corn grain, and alfalfa hay were estimated using the equation of Ørskov and McDonald (1979):

$$p = a + b\ (1 - e^{-ct})$$

where: p is the amount degraded (%) at time t (h); *a* is the soluble and/or rapidly degradable fraction (%); *b* is the potentially degradable fraction (%); and *c* is the rate of degradation of the potentially degradable fraction (% h^{-1}). An equation which included estimation of a lag phase (McDonald 1981) was used for barley straw:

$$p = a + b\ (1 - e^{-c(t-d)})$$

Where p, *a*, *b* and *c* are as defined above and *d* is the lag phase (h). These parameters were estimated by an iterative least square procedure using non-linear regression (Statistical Analysis System (SAS) 1985). The effective degradability of DM for barley grain, canola meal, corn grain, and alfalfa hay, of CP for canola meal (38.86% CP, DM basis) and alfalfa hay (15.82% CP, DM basis), and of NDF for alfalfa hay (46.50% NDF, DM basis), were calculated using the equation of Ørskov and McDonald (1979) as follows:

$$D = a + \frac{bc}{c + k}$$

where D is effective degradability (%), *a*, *b*, *c*, are as defined above, and k is the rumen turnover rate (h^{-1}). Effective degradabilities of DM, CP, and NDF were calculated using a rumen turnover rate of 0.05 h^{-1} (Agricultural Research Council (1984). Effective degradability of DM and NDF for barley straw (86.72% NDF, DM basis) were estimated by an equation which accounted for the estimated lag phase (Boila and Ingalls 1992).

Statistical analysis.

The study was a repeated 3x3 latin square design. Parameters *a*, *b*, *c* and *d*, effective degradability of DM for all feeds, effective degradability of CP for canola meal and alfalfa hay, effective degradability of NDF for alfalfa hay and barley straw, VFA and pH were subjected to analysis of variance using General Linear Models procedures of SAS (1985). Differences among treatment means were tested using Duncan's multiple range test.

Chemical analysis.

Feedstuff and fermentation residual N content was determined by the Micro-kjeldahl method (Method 47.023, Association of Official Analytical Chemists

(AOAC) 1984) using a Tecator 1030 analyzer (Tecator, AB Hoganas, Sweden). Neutral detergent fibre was determined using the procedure of Goering and Van Soest (1970) modified to exclude decalin and sodium sulphite. Acid detergent fibre in feedstuffs was determined by standard procedures (Method 7.076, AOAC 1984). Acid detergent insoluble nitrogen was determined on ADF residues using the Micro-kjeldahl procedure. Lignin and cellulose contents were determined by the method described by Robertson and Van Soest (1981). Hemicellulose was obtained as the difference between NDF and ADF (Robertson and Van Soest 1981). Calcium and phosphorus in the concentrate were determined following dry ashing (Method 7.101, AOAC 1984). Calcium was determined by atomic absorption spectroscopy (Method 7.096, AOAC 1984) and phosphorus was determined colorimetrically (Method 7.125, AOAC 1984). Estimation of fungal biomass was performed by determining the amount of glucosamine, using ion-exchange chromatography (Wittenberg et al. 1989). Total soluble carbohydrate content was determined following extraction in 80% (v/v) ethanol, using the procedure described by Jermyn (1975). Analysis of volatile fatty acids (VFA) was performed using gas chromatography as described by Erwin et al. (1961).

Forage samples were screened for mycotoxins. Trichothecene mycotoxins, namely, T-2 toxin, HT-2 toxin, diacetoxyscirpenol, deoxynivalenol, nivalenol, neosolaniol, fusarenon-X, trichothecin, roridin and verrucacin, were screened by a yeast bioassay (Madhyastha et al. 1994a). Non-trichothecene mycotoxins which include ochratoxin A, citrinin, patulin, penicillic acid, cyclopiazonic acid, penitren and zearalenone were screened using a *B. brevis* bioassay (Madhyastha et al. 1994b). Two subsamples for medium and high treatments were sent to the Veterinary Diagnostic Laboratory at North Dakota State University for verification of mycotoxin analysis.

Microbiological analysis

Approximately 15 ml of a sterile wash solution (0.1% Tween 80 solution) was added to a 2 g subsample of hay in sterile screwcap test tubes. The samples were shaken at 400 rpm for 10 min. Following removal from the shaker, the wash solution was removed and the residue placed into sterile centrifuge tubes and centrifuged at 7000 xg for 20 min. The supernatant was aspirated off and 2 ml of sterile wash solution added into each centrifuge tube to resuspend the pellet. The suspensions were then serially diluted using 0.1% Tween 80 solution and plated on Dichloran Rose Bengal agar (King et al. 1979) and incubated at 25°C for 5 days, after which the plates were enumerated and the major fungal species identified based on morphological characteristics (Pitt and Hocking 1985). Isolates were sent to the International Mycological institute (Biosystematics Services, Bakeham Lane, Surrey. UK) for verification. Counts were corrected to DM basis by drying the remaining forage samples in a forced-air oven at 60°C for 48 hours.

RESULTS AND DISCUSSION.

The LFB treatment was observed to be a green color with no visible mold. Bales used for MFB and HFB treatments were observed to be dusty and had visible mold in 50 to 100% of the bale. The LFB treatment would represent nonmoldy hay, while MFB and HFB treatments represented molded hay. Although treatments were formulated to be similar in nutrient profiles, lignin increased as glucosamine increased and water-soluble carbohydrate content decreased as glucosamine content increased.

Field fungi, namely, *Cladosporium* and *Phoma* spp. occured only in the LFB treatment (Fig. 18). Presence of field fungi in this treatment indicates that this forage did not heat to a large extent in storage. Field fungi persist in storage only if the forage is dry enough to prevent growth of typical storage fungi (Lacey 1989). Fifty percent of counts for MFB and HFB treatments were made up of unidentified thermotolerant hyphomycetes (Fig. 18) and efforts to have this fungus identifed were unsuccessful. Other common storage fungi which belong to the class Hyphomycetes include *A. fumigatus*, *Humicola lanuginosa*, *Paecilomyces variotti* and *Penicillium cyclopium* (Breton and Zwaenepoel 1991). *Aspergillus glaucus* and *A. versicolor* made up the remainder of the fungal counts in MFB and HFB treatments.

Forage treatments tested negative for mycotoxins. Screening for mycotoxins in studies where moldy hay is fed have not been done on a routine basis but it should be, considering that fungal species that occur in moldy hay are potential mycotoxin producers. Documentation of mycotoxins in hay is limited. A more clearly defined role of fungi in hay may be in depleting nutrients in forage, either in the field during wilting or during storage, rather than production of mycotoxins. However, the possibility of the occurrence of other unknown secondary metabolites cannot be totally excluded or discounted.

Hay intake was not significantly different ($P>0.05$) across forage treatments and averaged 2.1% of body weight (Table 18). Feeding moldy hay did not influence DM intake in studies with cattle or sheep (Mohanty et al. 1969; Smith et al. 1989; Wittenberg and Moshtagi-Nia 1990).

Concentrations of individual VFA or total VFA were not influenced ($P>0.05$) by different amounts of fungal biomass in hay (Table 18). Treatments also did not influence ($P>0.05$) the acetate to propionate ratio and rumen pH. Mohanty et al. (1969) reported that when steers were fed either clean or molded hay having similar crude fibre contents, individual VFA and total VFA were significantly higher on clean hay.

The rapidly soluble fraction (*a*) in each feed tested was not different ($P>0.05$) among forage treatments (Table 19). Barley straw had the lowest *a* fraction which averaged 7.0%. The *b* fraction of each test feed, similarly, was not significantly different ($P>0.05$) among treatments. Rates of DM degradation and effective degradability of DM for corn grain, barley grain, canola meal and alfalfa hay were not influenced ($P>0.05$) by treatment. However, rate of DM degradation and effective DM degradability for barley straw were higher ($P<0.05$) on the HFB treatment (Table 19).

The feeding of moldy hay has been reported to increase fibre digestibility of hay in other studies (Mohanty et al. 1969; Smith et al. 1989). The increased fibre digestibility was attributed to the action of molds on lignified fibre prior to feed ingestion, thereby making structural carbohydrate more available to rumen microbes (Mohanty et al. 1969). This is unlikely to be the case in the current study since fungal biomass in hay did not improve effective NDF degradability of barley straw. The role of fungal biomass in molded hay on degradation of straw in the rumen clearly merits further study.

Dietary fungal biomass concentration did not influence ($P>0.05$) either the *a* or *b* fractions, the rates of CP degradation in alfalfa hay and canola meal, or the effective CP degradability.(Table 20). Dietary treatments also did not influence ($P>0.05$) either the *a* or *b* fractions, the rates of NDF degradation and effective NDF degradability in alfalfa hay and barley straw.

These results are partially in agreement with those by others on the addition of direct-fed microbials, mainly yeast, *Saccharomyces cerevisiae,* and/or *Aspergillus oryzae* to dairy cattle diets. Selected strains of *S. cerevisiae* or *A. oryzae* did not alter the rumen environment as indicated by individual VFA and total VFA concentrations (Firkins et al. 1990; Gomez-Alarcon et al. 1990; Williams et al. 1991; Mutsvangwa et al. 1992; Piva et al. 1993). However, even with such defined microbial additions, results have been variable, since changes in ruminal acetate:propionate ratios and pH also have been reported (Wiedmeier et al. 1987; Williams et al. 1991; Piva et al. 1993). Erasmus et al. (1992) reported a tendency for higher DM disappearance of wheat straw and higher CP and NDF digestibility following supplementation with a yeast culture. One difference between direct-fed microbials (*A. oryzae* and *S. cerevisiae*) and fungal species in moldy hay is that the former can survive in anaerobic conditions and are, therefore, able to influence rumen fermentation directly. Fungal species in moldy hay are likely to affect fermantation by breaking structural bonds in forage cell wall prior to consumption or by production of enzymes or other substrates that may influence microbial action. Feeding moldy alfalfa hay to dairy steers did not affect the number of total protozoa even though there was a percent reduction in some species of protozoa (Mohanty et al. 1969). Rumen microbial species were not evaluated but it might be speculated that these were not influenced by fungal biomass in moldy hay.

CONCLUSION

Feeding moldy alfalfa hay does not appear to affect the rumen environment based on VFA and rumen pH measurements. This work shows that fungal biomass in molded alfalfa hay did not adversely interfere with DM, CP, or NDF digestion in commonly used starch, fiber and protein feed sources. However, rate and extent of DM degradation of highly lignified feedstuffs such as barley straw may be affected by presence of fungal biomass in hay. The role of fungi in hay seems mainly to be confined to their depletion of nutrients in forage during harvest and storage.

Figure 18. Predominant fungal species and counts following 25°C incubation in forage treatments having different amounts of fungal biomass. The forage treatments were low (LFB), medium (MFB) and high (HFB) amount of fungal biomass.

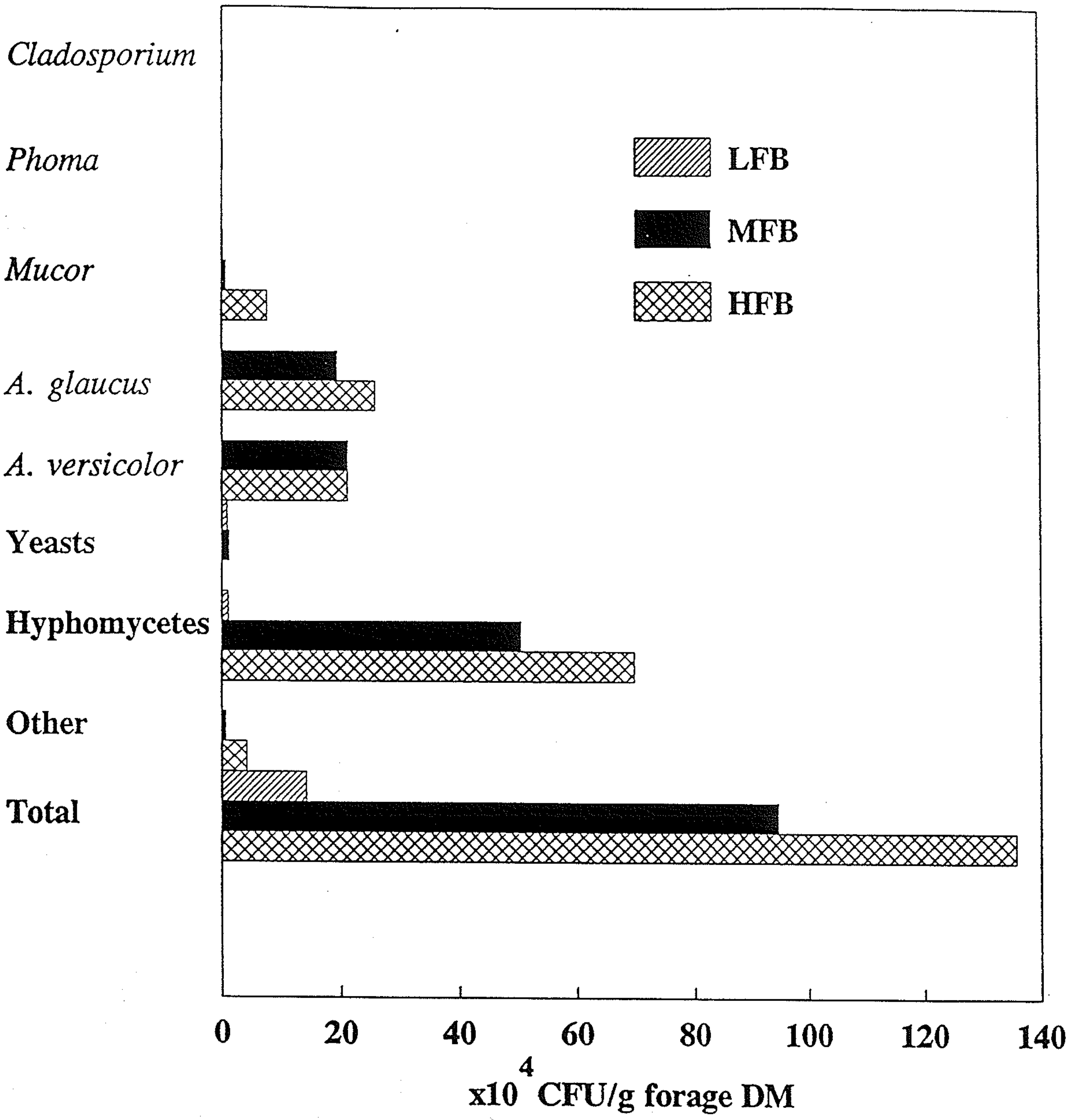

Cladosporium
Phoma
Mucor
A. glaucus
A. versicolor
Yeasts
Hyphomycetes
Other
Total
LFB
MFB
HFB
0
20
40
60
80
100
120
140
x10⁴ CFU/g forage DM

TABLE 16. Chemical composition of alfalfa forage treatments with three levels of fungal biomass.

	Forage treatment		
	LFB[X]	MFB	HFB
Dry matter, %	85.6	84.9	86.3
Crude protein, % DM	17.5	18.0	19.5
Neutral detergent fibre, %DM	47.9	49.3	48.3
Acid detergent fibre, %DM	34.1	35.5	37.2
Acid detergent insoluble N, %total N	5.6	6.1	8.4
Cellulose, % DM	25.5	27.5	26.8
Hemicellulose,% DM	13.8	13.8	11.1
Lignin, % DM	9.8	10.7	11.2
Total soluble carbohydrate, % DM	4.8	3.6	2.2
Mycotoxins[Y]	ND[Z]	ND	ND
Glucosamine, g/kg DM	1.6	2.5	3.3

[X] The forage treatments contained: low (LFB), medium (MFB) and high (HFB) fungal biomass concentrations.

[Y] Includes the following: T-2 toxin, HT-2 toxin, diacetoxyscirpenol, deoxynivalenol, nivalenol, neosolaniol, fusarenol-X, trichothecin, roridin, verrucacin, ochratoxin A, citrinin, patulin, penicillic acid, cyclopiazonic acid, penitren and zearalenone.

[Z] Not detected.

TABLE 17. Ingredients and chemical composition of the concentrate fed to lactating dairy cows.

Ingredient, % as-fed	
Rolled barley	73.2
Canola meal	14.0
Distiller grains	6.0
Beet molasses	4.0
Biophos[W]	0.8
Mineral premix[X]	0.8
Vitamin premix[Y]	0.6
Limestone	0.4
Selenium mix[Z] (200 mg kg^{-1})	0.2
Chemical composition, % as fed	
Crude protein	17.1
Neutral detergent fibre	32.9
Acid detergent fibre	8.3
Acid detergent insoluble N, % total N	8.7
Calcium	0.6
Phosphorus	0.8

[W] Contained 21% P and 18% Ca.

[X] Contained (mg kg^{-1}): potassium iodide 0.6, copper sulphate 15, zinc oxide 60.5, manganese oxide 61.2, magnessium oxide 0.65 g/kg and cobalt-iodized salt 6.6 g/kg.

[Y] Formulated to provide (IU kg^{-1}): Vit. A 15,000, Vit. D_3 3,000, and Vit. E 90.

[Z] Contained (%): Na selenite 0.045, mineral oil 1.5, rice hulls 40, and limestone 58.46.

TABLE 18. Effect of forage treatments with different amounts of fungal biomass on DM intake, ruminal VFA concentration, and pH.

	Forage treatment			
	LFB[z]	MFB	HFB	SE
Intake, kg DM d^{-1}	15.3	14.8	15.1	0.2
Intake, %BW	2.1	2.1	2.1	0.02
VFA, mmol l^{-1}				
Acetate (A)	71.8	74.9	72.3	2.9
Propionate (P)	16.3	17.9	15.7	1.1
Butyrate	13.9	14.7	14.3	0.7
Isobutyrate	1.8	2.1	1.7	0.1
Valerate	1.9	2.0	1.7	0.2
Isovalerate	2.1	2.4	1.9	0.2
Total	107.8	114.0	107.5	5.0
A:P	4.5	4.3	4.8	0.1
pH	6.4	6.4	6.5	0.2

[z] Forage treatments contained: low (LFB), medium (MFB) and high (HFB) fungal biomass concentrations.

TABLE 19. Dry matter disappearance parameters and effective degradabilties of test feeds when incubated in the rumen of dairy cows fed forage treatments with three levels of fungal biomass.

	Forage treatment			
	LFB[Z]	MFB	HFB	SE
a^X, %				
Corn grain	27.3	26.7	27.4	0.5
Barley grain	33.3	32.2	32.7	1.3
Canola meal	34.3	34.7	33.8	0.4
Alfalfa hay	31.8	30.9	30.7	0.3
Barley straw	7.4	6.7	6.8	0.1
b^X, %				
Corn grain	74.5	75.0	75.1	0.8
Barley grain	55.3	56.9	56.7	1.3
Canola meal	50.6	50.8	51.7	0.4
Alfalfa hay	39.4	41.7	41.6	0.6
Barley straw	39.8	41.4	38.9	0.6
c^X, % h^{-1}				
Corn grain	0.07	0.07	0.06	0.01
Barley grain	0.30	0.26	0.23	0.01
Canola meal	0.14	0.12	0.13	0.01
Alfalfa hay	0.11	0.11	0.10	0.01
Barley straw	0.022ab	0.017b	0.031a	0.001
Effective degradability[Y], %				
Corn grain	69.3	69.8	65.8	1.3
Barley grain	80.4	79.5	78.8	0.4
Canola meal	71.1	70.1	70.6	0.5
Alfalfa hay	58.8	58.8	57.4	0.6
Barley straw	17.9ab	17.1b	18.6a	0.02

a-bMeans within the same row with different letter differ ($P<0.05$).

[X] a=rapidly soluble and/or degradable fraction, b=potentially degradable fraction, c=rate of degradation of b.

[Y] Effective degradability at rumen outflow rate of 0.05 h^{-1}

[Z] Treatments contained: low (LFB), medium (MFB) and high (HFB) fungal biomass concentrations.

TABLE 20. Crude protein disappearance parameters and effective degradability of CP in alfalfa hay and canola meal in the rumen of dairy cows fed alfalfa forage with different levels of fungal biomass.

	Forage treatment			
	LFB[x]	MFB	HFB	SE
a[y], %				
Alfalfa hay	31.2	30.8	30.3	0.5
Canola meal	31.6	33.4	33.2	0,5
b[y], %				
Alfalfa hay	56.1	55.7	55.3	0.7
Canola meal	61.5	60.1	60.3	0.6
c[y], % h^{-1}				
Alfalfa hay	0.13	0.12	0.12	0.01
Canola meal	0.15	0.13	0.13	0.01
Effective degradability[z], %				
Alfalfa hay	71.6	69.4	69.3	0.7
Canola meal	77.6	76.4	76.8	0.5

[x] Treatments contained: low (LFB), medium (MFB) and high (HFB) fungal biomass concentrations.

[y] *a*=rapidly soluble and/or degradable fraction, *b*=potentially degradable fraction, and *c*=rate of degradation of *b*.

[z] Effective degradability at rumen outflow rate of 0.05 h^{-1}

TABLE 21. Neutral detergent fibre disappearance parameters and effective degradability of NDF in alfalfa hay and barley straw in the rumen of cows fed alfalfa forage with three levels of fungal biomass.

	Forage treatment			
	LFB[x]	MFB	HFB	SE
a[y], %				
Alfalfa hay	29.4	29.1	27.4	0.4
Barley straw	10.3	10.3	10.4	0.2
b[y], %				
Alfalfa hay	33.7	34.5	36.3	0.5
Barley straw	40.3	40.9	36.4	1.1
c[y], % h^{-1}				
Alfalfa hay	0.09	0.08	0.09	0.01
Barley straw	0.02	0.02	0.02	0.001
Effective degradability[z], %				
Alfalfa hay	51.0	49.9	49.9	0.8
Barley straw	19.3	18.8	19.5	0.2

[x] Treatments contained low (LFB), medium (MFB) and high (HFB) fungal biomass concentration.

[y] *a*=rapidly soluble and/or degradable fraction, *b*=potentially degradable fraction, *c*=rate of degradation of *b*.

[z] Effective degradability at rumen outflow rate of 0.05 h^{-1}

GENERAL DISCUSSION.

A better understanding of events during harvest and storage of alfalfa forage would lead to more informed decisions as to optimum harvest time and also appropriate DM contents at which to store forage. The other question to address is whether fungal biomass in moldy hay should be of concern regarding preference of the hay and digestion of other feeds in a ration.

Storage losses of DM and nutrients are influenced by initial DM content, being higher in low DM forage (manuscript I). The highest DM content at which forage was stored was 75.4-77.4%, which is lower than the 80-85% currently recommended for long term storage of small square bales (Jones and Harris 1980; Robertson 1983). Based on the extent of DM and nutrient losses, losses in nutritional value, microbial species active during storage, and storage temperatures, the feasibility of baling and storing alfalfa forage at DM contents as low as 75% should be re-examined. An interesting observation from the current study was that nutritional composition changes occur during periods when temperature is high during storage. During this period, rate of increase of DM, NDF, ADF, ADIN, and glucosamine was higher in low DM forage relative to high DM forages. Forage baled at the lowest DM content had the highest peak temperature during storage. The increase in ADIN concentration in stored hay does not appear to be a function of temperature alone. A part of the N associated with fungal cell wall may contribute to the changes observed in forage ADIN concentration.

Moisture content at baling was important in determining which fungal species were predominant at the end of storage. (Manuscript II). During the early storage phase, *Cladosporium, Alternaria* , *Phoma* spp. and yeasts, were present in all forages. Storage at a high DM content resulted in predominance of *A. repens*, and *Absidia* spp., while at low DM contents, *A. fumigatus*, *E. nidulans*, *Absidia* and *Mucor* spp. and unidentified thermotolerant hyphomycetes were more dominant.

European studies have described fungal succession in grass hay, from those which are considered field fungi to storage fungi (Gregory et al. 1963; Kasspersson et al. 1984; Breton and Zwaenepoel 1991). There has been no detailed work has been reported in alfalfa forage under North American conditions, to date and the work presented here (Manuscript II) is the first attempt to document detailed fungal succession during storage of alfalfa forage. This study demonstrated that when alfalfa forage was stored at DM contents of 71.9 to 77.4%, *A. repens*, and *Absidia* spp. are the predominant fungal species. Unidentified thermotolerant hyphomycetes were dominant in forage stored at lower DM contents. There is a definite need to identify this fungal species since they are dominant in stored low DM alfalfa hay.

Studies on hay storage have either concentrated on nutrient changes during storage or changes in fungal species in isolation, with no attempts to relate the two aspects. This work has attempted to relate changes in forage constituents with microbial changes and temperature in the initial 60 days of hay storage. The goal in hay-making should, therefore, be to suppress fungal growth early, which can be achieved by accelerating rate of moisture loss during storage to levels that do not support fungal growth. Storage at a low DM content resulted in high counts of species such as *A. fumigatus* which has been implicated in health problems. Alfalfa forage stored at 75.4-77.5% DM in contrast resulted in minimum nutrient losses and although fungal growth was evident, the predominant species have not been reported to be associated with major health problems.

A hay grading system requires or should require an evaluation of both, nutrient content of hay and assessment for mold and color. Our study showed that the use of visual mold assessment is very limited. This introduces bias in hay grading, which can lead to unfair trading or even reluctance by producers to have their haylots graded, a problem encountered during the course of these studies. It has been demonstrated by a number of workers that glucosamine, a component of fungal cell wall can be used as an estimate of fungal biomass in different agricultural products. This method has been used to evaluate the fungal biomass status of grain and oilseeds (Donald and Mirocha 1977; Nandi 1978; Rotter et al. 1989) and hay (Jones et al. 1985; Roberts et al. 1987a; Wittenberg et al. 1989). Such a measure reduces subjectivity to a large extent and makes use of NIR technology a feasible option for use in hay grading and marketing. Potential for use of NIRS to estimate fungal biomass in hay was demonstrated earlier by Roberts et al. (1987b). The current study, which incorporated a wider range of samples covering a period of 5 years and different harvest and storage conditions, demonstrated that NIRS can be used to establish hay fungal biomass on a commercial scale (Manuscript III). Glucosamine detection using NIRS is not limited to a few wavelengths and instrumentation capability will ultimately determine wavelngths used.

The quality of hay is ultimately determined by animal performance. Previous research has shown that there are no differences in intake between moldy and nonmoldy hay when they were available as the only feed (Smith et al. 1989; Wittenberg and Moshtaghi-Nia 1990; Bossuyt 1995). The objective was to examine whether hay preference would be affected when hays with similar nutrient profiles but differing in amounts of fungal biomass were offered to calves with no previous exposure to moldy feedstuffs (manuscript IV). This work showed that preference is reduced by fungal biomass in moldy hay.

In dairy cattle rations alfalfa hay can be fed as the sole fibre source and hay quality in such situations is important. A further objective was to examine whether fungal biomass in moldy hay affects digestion kinetics of DM, CP and NDF in other feedstuffs. Intake of hay with increasing amounts of fungal biomass did not adversely affect the rate and extent of ruminal DM, CP or NDF degradation of commonly used starch, protein and fibre feed sources (Manuscript V). Interpretation of these results should be made keeping in mind that fungal species in hay can exert an indirect influence on rumen fermentation by utilizing some nutrients during storage and/or producing products that might affect the rumen microflora.

FUTURE CONSIDERATIONS

Laboratories are likely to do species identification to establish possible health hazards due to spores and mycotoxins in molded hays. The work reported here shows that there is a great deal of variability in fungal species composition during storage, and hence, laboratory analysis may be misleading. Hay samples taken after storage do not show the history of fungal species presence and absence. If a species is not present at the end of storage, it does not necessarily mean that the species was never present or that its by-products (spores and mycotoxins) are not present. Temperatures reached during storage might give an indication as to the possible presence of infection-causing fungal species in hay, since thermotolerant fungal species are likely to be associated with mycosis in warm-blooded animals.

The work reported here also suggests the need to re-evaluate "safe" DM contents for storage. Under adverse weather conditions, it might be advisable to avoid field losses such as those due to leaching and field molding by baling and storing forage at lower DM contents. Storage losses were minimal when alfalfa forage was stored at DM contents of 75.4-77.4%. Whether presence of fungal biomass in moldy hay should result in a lower price for hay is debatable. This should depend on the amount of fungal biomass present and (suspected) storage conditions. Any indication of how much the forage heated during storage (eg. using hay color) would be helpful in this respect and would lead to some suspicion about the possible existence of thermophilic fungi. Some molding, especially at higher DM content such as that described in this work will not reduce the feed value of hay. In the final analysis, nutrient content should be the basis for pricing of hay.

The challenge to researchers working in forage conservation should focus on:

1. Methods to improve forage wilting rate to minimize field DM and nutrient losses. Until greater control can be achieved during harvest and storage, research on finding optimum ways of feeding moldy hay is necessary and important.

2. More extensive screening for mycotoxins in moldy hay. In this current work, forages used in feeding studies were always screened for mycotoxins and none were found. However, studies on hay storage rarely look at this aspect and there is little documentation of mycotoxin presence in hay. Future studies should focus on storing forage for long periods, one year, or more and under different weather conditions to evaluate whether mycotoxins will occur in hay.

3. The role of hay preservatives, how they affect fungal succession and nutrient changes in the early phases of storage would give an insight of their effectiveness and mode of action. This is especially important for bacterial additives, whose mode of action has not been elucidated as yet.

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Appendix IA: ANOVA Tables for changes in forage constituents during storage (Manuscript I).

Dependent variable: DM

Source	DF	SS	MS	F value	Pr>F
Trt	2	400.92	200.46	53.63	0.0001
Stack(Trt)	3	11.83	3.94	1.05	0.383
Bale(Trt*Stack)	6	85.33	14.22	3.81	0.0062
Day	5	2242.75	448.55	120.01	0.0001
Trt*Day	10	164.14	16.41	4.39	0.0008
Stack*Day(Trt)	15	23.39	1.56	0.42	0.962
Error	30	112.13	3.74		

Dependent variable: NDF

Source	DF	SS	MS	F value	Pr>F
Trt	2	89.94	44.97	13.43	0.0001
Stack(Trt)	6	5.49	1.83	0.55	0.654
Bale(Trt*Stack)	3	35.50	5.91	1.77	0.140
Day	5	159.23	31.85	9.51	0.0001
Trt*Day	10	85.05	8.51	2.54	0.024
Stack*Day(Trt)	15	65.27	4.35	1.30	0.262
Error	30	100.45	3.35		

Dependent variable: ADF

Source	DF	SS	MS	F value	Pr>F
Trt	2	41.78	20.89	7.36	0.0025
Stack(Trt)	3	18.55	6.18	2.18	0.111
Bale(Trt*Stack)	6	21.00	3.50	1.23	0.317
Day	5	124.41	24.88	8.77	0.0001
Trt*Day	10	43.04	4.30	1.52	0.182
Stack*Day(Trt)	15	53.41	3.56	1.26	0.288
Error		85.11	2.84		

Dependent variable: CP

Source	DF	SS	MS	F value	Pr>F
Trt	2	10.40	5.20	8.18	0.0015
Stack(Trt)	3	14.07	4.69	7.38	0.0008
Bale(Trt*Stack)	6	3.58	0.60	0.94	0.482
Day	5	24.41	4.88	7.68	0.0001
Trt*Day	10	5.33	0.53	0.84	0.596
Stack*Day(Trt)	15	31.32	2.09	3.28	0.0027
Error	30	19.08	0.64		

Dependent variable: ADIN

Source	DF	SS	MS	F value	Pr>F
Trt	2	30.62	15.31	124.17	0.0001
Stack(Trt)	3	0.56	0.19	1.52	0.230
Bale(Trt*Stack)	6	3.27	0.55	4.42	0.003
Day	5	38.78	7.76	62.91	0.0001
Trt*Day	10	16.98	1.70	13.77	0.0001
Stack*Day(Trt)	15	11.15	0.74	6.03	0.0001
Error	30	3.70	0.12		

Appendix IB: ANOVA Tables for forage constituents.

Dependent variable: cellulose

Source	DF	SS	MS	F value	Pr>F
Trt	2	13.12	6.56	4.45	0.020
Stack(Trt)	3	8.34	2.78	1.89	0.153
Bale(Trt*Stack)	6	4.76	0.79	0.54	0.775
Day	5	46.38	9.28	6.30	0.0004
Trt*Day	10	14.40	1.44	0.98	0.483
Stack*Day(Trt)	15	25.02	1.67	1.13	0.372
Error	30	44.19	1.47		

Dependent variable: water-soluble carbohydrate

Source	DF	SS	MS	F value	Pr>F
Trt	2	8.21	4.10	9.93	0.0005
Stack(Trt)	3	0.72	0.24	0.58	0.635
Bale(Trt*Stack)	6	1.61	0.27	0.65	0.690
Day	5	107.72	21.54	52.12	0.0001
Trt*Day	10	6.59	0.66	1.59	0.156
Stack*Day(Trt)	15	2.57	0.17	0.41	0.963
Error	30	12.40	0.41		

Dependent variable: glucosamine

Source	DF	SS	MS	F value	Pr>F
Trt	2	5.76	2.88	23.97	0.0001
Stack(Trt)	3	0.10	0.03	0.28	0.841
Bale(Trt*Stack)	6	0.93	0.16	1.29	0.291
Day	5	19.54	3.91	32.55	0.0001
Trt*Day	10	3.74	0.37	3.11	0.0077
Stack*Day(Trt)	15	0.87	0.06	0.48	0.932
Error	30	3.60	0.12		

Dependent variable: lignin

Source	DF	SS	MS	F value	Pr>F
Trt	2	9.01	4.50	17.11	0.0001
Stack(Trt)	3	2.29	076	2.90	0.051
Bale(Trt*Stack)	6	4.29	0.71	2.72	0.032
Day	5	27.68	5.54	21.04	0.0001
Trt*Day	10	3.69	0.37	1.40	0.227
Stack*Day(Trt)	15	7.73	0.52	1.96	0.057
Error	30	7.90	0.26		

Appendix IIA: ANOVA Tables for fungal data in manuscript II.

Dependent variable: Total fungal counts for the whole storage period

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	24.81	12.41	1.62	0.252
Error	9	69.14	7.68		

Within Trts

Source	DF	SS	MS	F value	Pr>F
Days	12	301.90	25.16	3.19	0.001
Day*Trt	24	288.76	12.03	1.53	0.074
Error	108	850.95	7.88		

Dependent variable: Total fungal counts (days 1-8)

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	34.91	17.46	1.25	0.332
Error	9	125.91	13.99		

Within Trts

Source	DF	SS	MS	F value	Pr>F
Day	7	86.04	12.29	1.33	0.25
Day*Trt	14	130.68	9.33	1.01	0.45
Error	63	583.78	9.27		

Dependent variable: Total fungal counts (days 9-60)

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	124.26	62.13	9.26	0.007
Error	9	60.38	6.71		

Within Trts

Source	DF	SS	MS	F value	Pr>F
Day	4	28.28	7.07	1.70	0.18
Day*Trt	8	23.72	2.97	0.711	0.65
Error	36	150.02	4.17		

Dependent variable: Number of species (days 1-60)

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	31.34	15.67	2.15	0.173
Error	9	65.66	7.30		

Within Trts

Source	DF	SS	MS	F value	Pr>F
Day	12	72.14	6.01	1.87	0.04
Day*Trt	24	155.83	6.49	2.02	0.001
Error	108	347.76	3.22		

Dependent variable: number of species (days 1-8)

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	64.40	32.20	4.20	0.051
Error	9	68.93	7.66		

Within Trts

Source	DF	SS	MS	F value	Pr>F
Day	7	54.58	7.80	2.073	0.06
Day*Trt	14	65.47	4.68	1.243	0.26
Error	63	237.03	3.76		

Dependent variable: number of species (days 9-60)

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	26.70	13.35	4.64	0.041
Error	9	25.88	2.88		

Within Trts

Source	DF	SS	MS	F value	Pr>F
Day	4	16.44	4.11	1.81	0.14
Day*Trt	8	30.60	3.83	1.69	0.13
Error	36	81.57	2.27		

Appendix IIB: (Cont'd): Anova Tables for fungal data.

Dependent variable: Log series α index (days 1-60).

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.12	0.061	0.578	0.589
Error	9	0.64	0.11		

Within Trts

Source	DF	SS	MS	F value	Pr>F
Day	12	0.83	0.070	2.039	0.03
Day*Trt	24	0.51	0.021	0.627	0.89
Error	72	2.46	0.034		

Dependent variable: Log series α index (days 1-8)

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.15	0.07	0.725	0.522
Error	6	0.61	0.10		

Within Trts

Source	DF	SS	MS	F value	Pr>F
Day	7	0.46	0.065	1.55	0.17
Day*Trt	14	0.25	0.018	0.43	0.95
Error	42	1.76	0.042		

Dependent variable: Log series α (days 9-60)

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.059	0.029	0.772	0.491
Error	9	0.342	0.038		

Within Trts

Source	DF	SS	MS	F value	Pr>F
Day	4	0.084	0.021	1.21	0.32
Day*Trt	8	0.25	0.031	1.77	0.11
Error	36	0.62	0.017		

Dependent variable: Berger-Parker index (days 1-8)

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.099	0.050	0.61	0.574
Stack(Trt)	3	0.44	0.15	1.81	0.246
Error	6	0.49	0.08		

Within Trts

Day	7	0.66	0.09	1.38	0.241
Day*Trt	14	1.28	0.09	1.34	0.225
Day*Stack(Trt)	21	1.84	0.08	1.28	0.241
Error	42	2.87	0.07		

Dependent variable: Berger-Parker index (days 9-60)

Between Trts

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.62	0.31	6.01	0.037
Stack(Trt)	3	0.12	0.040	0.79	0.543
Error	6	0.31	0.051		

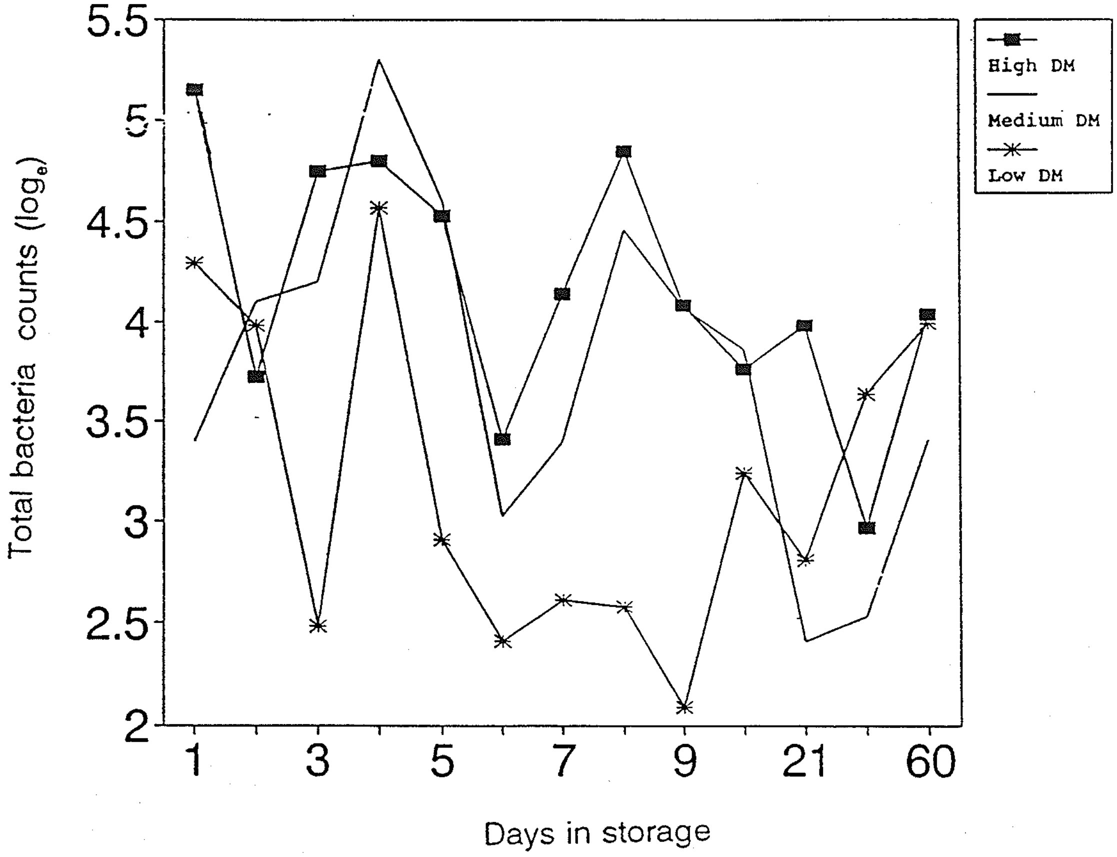
Within Trts

Day	4	0.29	0.073	1.77	0.17
Day*Trt	8	0.17	0.022	0.53	0.82
Day*Stack(Trt)	12	0.55	0.046	1.11	0.40
Error	24	0.98	0.041		

Appendix IIC: Anova Table for total bacterial counts and graph showing changes in total bacterial counts during storage (Manuscript II).

Dependent variable: Total bacterial counts

Source	DF	SS	MS	F value	Pr>F
Between Trts					
Trt	2	23.71	11.85	6.54	0.031
Stack(Trt)	3	9.93	1.82	1.82	0.243
Error	6	10.88	1.81		
Within Trts					
Day	12	56.93	4.74	2.57	0.0117
Day*trt	24	40.54	1.69	0.92	0.569
Day*stack(Trt)	36	88.28	2.45	1.33	0.173
Error	72	132.69	1.84		



Appendix III: ANOVA Table for the hay preference study (Manuscript IV).

Dependent variable: Consumed

Source	DF	SS	MS	F value	Pr>F
Model	54	1424.11	26.37	5.85	0.0001
Error	87	392.40	4.51		
Total	141	1816.51			
Source	DF	SS	MS	F value	Pr>F
Animal	5	52.71	10.54	2.34	0.049
Period	5	517.34	103.47	22.94	0.0001
Pair	5	88.64	17.73	3.93	0.0029
Square	1	102.44	102.44	22.71	0.0001
Anim(square)	5	29.72	5.94	1.32	0.264
Per(square)	5	28.63	5.73	1.27	0.284
Anim*Per	25	38.63	1.55	0.34	0.998
Feed	3	618.05	206.02	45.68	0.0001

Appendix IVA: ANOVA Tables for volatile fatty acids (Manuscript V).

Dependent variable: Propionate

Source	DF	SS	MS	F value	Pr>F
Trt	2	15.97	7.99	0.36	0.710
Per(sq)	5	84.18	16.84	0.76	0.609
Cow	2	9.27	4.64	0.21	0.817
Sq*cow	2	10.60	5.30	0.24	0.794
Error	6	132.60	22.10		

Dependent variable: Acetate

Source	DF	SS	MS	F value	Pr>F
Trt	2	33.97	16.98	0.11	0.896
Per(sq)	5	170.55	34.10	0.23	0.938
Cow	2	116.73	58.37	0.39	0.695
Sq*cow	2	26.10	13.05	0.09	0.918
Error	6	906.29	151.05		

Dependent variable: Isobutyrate

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.51	0.26	1.83	0.239
Per(sq)	5	0.56	0.11	0.80	0.590
Cow	2	0.47	0.23	1.65	0.269
Sq*cow	2	1.08	0.54	3.87	0.083
Error	6	0.84	0.14		

Dependent variable: Butyrate

Source	DF	SS	MS	F value	Pr>F
Trt	2	2.03	1.02	0.10	0.905
Per(sq)	5	60.57	12.11	1.22	0.403
Cow	2	31.95	15.98	1.60	0.277
Sq*cow	2	11.86	5.93	0.60	0.581
Error	6	59.78	9.96		

Dependent variable: Isovalerate

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.69	0.35	0.97	0.431
Per(sq)	5	0.54	0.11	0.30	0.895
Cow	2	0.58	0.29	0.82	0.483
Sq*cow	2	0.46	0.23	0.65	0.557
Error	6	2.14	0.36		

Dependent variable: Valerate

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.44	0.22	0.54	0.606
Per(sq)	5	2.33	0.47	1.16	0.423
Cow	2	0.66	0.33	0.82	0.483
Sq*cow	2	1.17	0.58	1.45	0.306
Error	6	2.41	0.40		

Dependent variable: Total VFA

Trt	2	164.25	82.12	0.19	0.831
Per(sq)	5	889.64	177.93	0.41	0.824
Cow	2	455.72	227.86	0.53	0.613
Sq*cow	2	56.96	28.48	0.07	0.936
Error	6	2573.50	428.92		

Appendix IVB: pH, intake and intake as %BW.

Dependent Variable: pH

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.059	0.030	3.02	0.124
Per(sq)	5	0.076	0.015	1.56	0.301
Cow	2	0.064	0.032	3.24	0.111
Sq*cow	2	0.012	0.006	0.62	0.567
Error	6	0.059	0.010		

Dependent variable: Intake

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.73	0.37	0.78	0.501
Per(sq)	5	16.26	3.25	6.91	0.0178
Cow	2	101.80	50.90	108.25	0.0001
Sq*cow	2	0.13	0.07	0.14	0.873
Error	6	2.82	0.47		

Dependent variable: Intake as % BW

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.016	0.008	0.84	0.478
Per(sq)	5	0.32	0.065	6.72	0.019
Cow	2	3.68	1.84	191.06	0.0001
Sq*cow	2	0.0005	0.0003	0.03	0.972
Error	6	0.058	0.0096		

Appendix IVC: ANOVA Tables for DM disappearance parameters for barley and corn.

Dependent variable: a

Source	DF	SS	MS	F value	Pr>F
Trt	2	3.44	1.72	0.06	0.942
Per(sq)	5	86.68	17.34	0.61	0.699
Cow	2	5.94	2.97	0.10	0.903
Sq*cow	2	78.69	39.34	1.38	0.321
Error	6	170.97	28.49		

Dependent variable: b

Source	DF	SS	MS	F value	Pr>F
Trt	2	8.62	4.31	0.14	0.876
Per(sq)	5	70.68	14.14	0.45	0.803
Cow	2	11.63	5.81	0.18	0.837
Sq*cow	2	43.16	21.58	0.68	0.541
Error	6	190.16	31.69		

Dependent variable: c

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.015	0.008	2.43	0.169
Per(sq)	5	0.038	0.008	2.43	0.155
Cow	2	0.023	0.011	3.59	0.095
Sq*cow	2	0.002	0.001	0.39	0.693
Error	6	0.019	0.003		

Dependent variable: Effective degradability

Source	DF	SS	MS	F value	Pr>F
Trt	2	7.42	3.71	1.56	0.284
Per(sq)	5	35.16	7.03	2.96	0.109
Cow	2	4.11	2.06	0.87	0.467
Sq*cow	2	7.24	3.62	1.52	0.292
Error	6	14.24	2.37		

Dependent variable: a (for corn DM parameters).

Source	DF	SS	MS	F value	Pr>F
Trt	2	1.78	0.89	0.20	0.824
Per(sq)	5	81.59	16.32	3.68	0.072
Cow	2	22.80	11.40	2.57	0.156
Sq*cow	2	26.22	13.11	2.96	0.128
Error	6	26.59	4.43		

Dependent variable: b (corn)

Source	DF	SS	MS	F value	Pr>F
Trt	2	1.45	0.73	0.07	0.934
Per(sq)	5	80.81	16.16	1.54	0.305
Cow	2	54.87	27.43	2.62	0.152
Sq*cow	2	15.83	7.92	0.75	0.510
Error	6	62.94	10.49		

Dependent variable: c

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.0004	0.0002	0.41	0.684
Per(sq)	5	0.0034	0.0007	1.29	0.379
Cow	2	0.0001	0.00007	0.13	0.882
Sq*cow	2	0.00004	0.00002	0.03	0.968
Error	6	0.003	0.0005		

Dependent variable: Effective degradability (for corn)

Source	DF	SS	MS	F value	Pr>F
Trt	2	58.86	29.43	0.94	0.443
Per(sq)	5	256.32	51.26	1.63	0.283
Cow	2	20.60	10.30	0.33	0.733
Sq*cow	2	4.24	2.12	0.07	0.936
Error	6	188.74	31.45		

Appendix IVD: ANOVA Tables for alfalfa DM and CP disappearance parameters (Manuscript V).

Dependent variable: a (for alfalfa DM disappearance)

Source	DF	SS	MS	F value	Pr>F
Trt	2	3.72	1.86	0.96	0.436
Per(sq)	5	261.73	52.35	26.92	0.0005
Cow	2	30.17	15.08	7.76	0.0217
Sq*cow	2	6.32	3.16	1.62	0.273
Error	6	11.67	1.94		

Dependent variable: b

Source	DF	SS	MS	F value	Pr>F
Trt	2	20.19	10.09	1.81	0.242
Per(sq)	5	159.80	31.96	5.74	0.028
Cow	2	32.99	16.50	2.96	0.127
Sq*cow	2	2.56	1.28	0.23	0.801
Error	6	33.40	5.57		

Dependent variable: c

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.0003	0.0001	0.25	0.786
Per(sq)	5	0.0062	0.0012	2.16	0.187
Cow	2	0.0015	0.0007	1.31	0.338
Sq*cow	2	0.0008	0.0004	0.74	0.515
Error	6	0.0034			

Dependent variable: Effective degradability

Source	DF	SS	MS	F value	Pr>F
Trt	2	7.64	3.82	0.68	0.541
Per(sq)	5	153.69	30.74	5.49	0.031
Cow	2	16.13	8.07	1.44	0.309
Sq*cow	2	21.37	10.69	1.91	0.228
Error	6	33.61	5.60		

Dependent variable: a (for alfalfa cp disappearance)

Source	DF	SS	MS	F value	Pr>F
Trt	2	2.43	1.22	0.29	0.761
Per(sq)	5	116.39	23.28	5.49	0.031
Cow	2	10.56	5.28	1.24	0.353
Sq*cow	2	17.40	8.70	2.05	0.209
Error	6	25.46	4.24		

Dependent variable: b

Source	DF	SS	MS	F value	Pr>F
Trt	2	1.89	0.94	0.12	0.891
Per(sq)	5	219.99	44.00	5.47	0.038
Cow	2	2.10	1.05	0.13	0.880
Sq*cow	2	17.87	8.94	1.11	0.389
Error	6	48.30	8.05		

Dependent variable: c

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.0006	0.0003	0.39	0.693
Per(sq)	5	0.0021	0.0004	0.54	0.744
Cow	2	0.0007	0.0004	0.49	0.637
Sq*cow	2	0.0002	0.0001	0.14	0.869
Error	6	0.0046	0.0008		

Dependent variable: Effective CP degradability

Source	DF	SS	MS	F value	Pr>F
Trt	2	20.71	10.35	1.18	0.369
Per(sq)	5	150.38	30.08	3.44	0.082
Cow	2	5.65	2.83	0.32	0.736
Sq*cow	2	2.61	1.31	0.15	0.865
Error	6	52.51	8.75		

Appendix IVE: ANOVA Tables for alfalfa NDF and canola DM disappearance parameters.

Dependent variable: a (alfalfa NDF)

Source	DF	SS	MS	F value	Pr>F
Trt	2	14.60	7.30	3.13	0.117
Per(sq)	5	272.61	54.52	23.37	0.0007
Cow	2	13.94	6.97	2.99	0.126
Sq*cow	2	3.65	1.82	0.78	0.499
Error	6	13.99	2.33		

Dependent variable: b

Source	DF	SS	MS	F value	Pr>F
Trt	2	20.93	10.47	2.31	0.180
Per(sq)	5	31.95	3.39	1.14	0.340
Cow	2	9.66	4.83	1.07	0.401
Sq*cow	2	12.26	6.13	1.36	0.327
Error	6	27.15	4.52		

Dependent variable: c

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.0008	0.0004	0.51	0.626
Per(sq)	5	0.0042	0.0008	1.09	0.453
Cow	2	0.00006	0.00003	0.04	0.963
sq*cow	2	0.00052	0.00026	0.34	0.727
Error	6				

Dependent variable: Effective degradability

Source	DF	SS	MS	F value	Pr>F
Trt	2	4.77	2.38	0.23	0.798
Per(sq)	5	326.73	65.35	6.41	0.021
Cow	2	11.20	5.60	0.55	0.604
Sq*cow	2	7.55	3.78	0.37	0.705
Error	6	61.14	10.19		

Dependent variable: a (for canola DM disappearance)

Source	DF	SS	MS	F value	Pr>F
Trt	2	2.29	1.14	0.33	0.733
Per(sq)	5	156.60	31.32	8.96	0.009
Cow	2	25.21	12.61	3.61	0.094
Sq*cow	2	29.37	14.69	4.20	0.072
Error	6	20.97	3.49		

Dependent variable: b

Source	DF	SS	MS	F value	Pr>F
Trt	2	4.10	2.05	0.66	0.553
Per(sq)	5	132.86	26.57	8.49	0.011
Cow	2	43.04	21.52	6.88	0.028
Sq*cow	2	33.40	16.70	5.34	0.045
Error	6	18.77	3.13		

Dependent variable: c

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.0008	0.0004	0.76	0.506
Per(sq)	5	0.029	0.006	11.56	0.005
Cow	2	0.0011	0.0005	1.10	0.391
Sq*cow	2	0.0006	0.0003	0.64	0.559
Error	6	0.003	0.0005		

Dependent variable: Effective degradability

Source	DF	SS	MS	F value	Pr>F
Trt	2	3.50	1.75	0.46	0.651
Per(sq)	5	80.64	16.13	4.25	0.053
Cow	2	16.73	8.37	2.21	0.191
Sq*cow	2	12.53	6.26	1.65	0.268
Error	6	22.76	3.79		

Appendix IVF: ANOVA Tables for canola CP and straw DM disappearance parameters.

Dependent variable: a (for canola CP)

Source	DF	SS	MS	F value	Pr>F
Trt	2	11.14	5.57	1.15	0.379
Per(sq)	5	79.20	15.84	3.26	0.091
Cow	2	5.53	2.77	0.57	0.594
Sq*cow	2	2.31	1.15	0.24	0.796
Error	6	29.17	4.86		

Dependent variable: b

Source	DF	SS	MS	F value	Pr>F
Trt	2	6.79	3.39	0.55	0.601
Per(sq)	5	121.74	24.35	3.98	0.062
Cow	2	5.60	2.80	0.46	0.654
Sq*cow	2	4.46	2.23	0.36	0.709
Error	6	36.75	6.12		

Dependent variable: c

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.001	0.0007	1.84	0.238
Per(sq)	5	0.003	0.0005	1.49	0.318
Cow	2	0.003	0.002	4.27	0.070
Sq*cow	2	0.0008	0.0004	1.07	0.400
Error	6	0.002	0.0004		

Dependent variable: Effective degradability

Source	DF	SS	MS	F value	Pr>F
Trt	2	4.69	2.34	0.56	0.599
Per(sq)	5	27.50	5.50	1.13	0.372
Cow	2	14.28	7.14	1.70	0.260
Sq*cow	2	7.60	3.80	0.90	0.454
Error	6	25.21	4.20		

Dependent variable: a (for straw DM disappearance

Source	DF	SS	MS	F value	Pr>F
Trt	2	1.66	0.83	2.48	0.164
Per(sq)	5	13.49	2.70	10.08	0.008
Cow	2	1.98	0.99	2.97	0.127
Sq*cow	2	1.90	0.95	2.84	0.136
Error	6	2.00	0.33		

Dependent variable: b

Source	DF	SS	MS	F value	Pr>F
Trt	2	16.14	8.07	1.09	0.404
Per(sq)	5	36.02	7.20	1.22	0.407
Cow	2	25.68	12.84	1.74	0.267
Sq*cow	2	2.37	1.19	0.16	0.856
Error	6	36.88	7.38		

Dependent variable: c

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.00005	0.00002	2.80	0.045
Per(sq)	5	0.00011	0.00002	3.10	0.123
Cow	2	0.000008	0.000004	0.46	0.656
Sq*cow	2	0.00001	0.00001	1.53	0.302
Error	6	0.00004	0.000009		

Dependent variable: d (lag)

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.014	0.0068	0.01	0.994
Per(sq)	5	3.768	0.754	0.78	0.581
Cow	2	1.366	0.683	0.57	0.599
Sq*cow	2	0.0587	0.029	0.02	0.976
Error	6	6.007	1.201		

Appendix IVG: ANOVA Tables for straw DM and NDF disappearance.

Dependent variable: Effective DM degradability

Source	DF	SS	MS	F value	Pr>F
Trt	2	1.62	0.81	1.54	0.036
Per(sq)	5	12.90	2.58	6.13	0.301
Cow	2	2.85	1.43	2.71	0.159
Sq*cow	2	4.43	2.22	4.21	0.085
Error	6	2.63	0.53		

Dependent variable: a (for straw NDF)

Source	DF	SS	MS	F value	Pr>F
Trt	2	0.02	0.01	0.02	0.979
Per(sq)	5	5.42	1.08	2.23	0.178
Cow	2	0.50	0.25	0.51	0.624
Sq*cow	2	1.94	0.97	2.00	0.216
Error	6	2.91	0.49		

Dependent variable: b

Source	DF	SS	MS	F value	Pr>F
Trt	2	70.85	35.43	1.39	0.318
Per(sq)	5	159.39	31.88	1.25	0.390
Cow	2	69.79	34.89	1.37	0.323
Sq*cow	2	5.63	2.82	0.11	0.897
Error	6	152.69	25.45		

Dependent variable: c

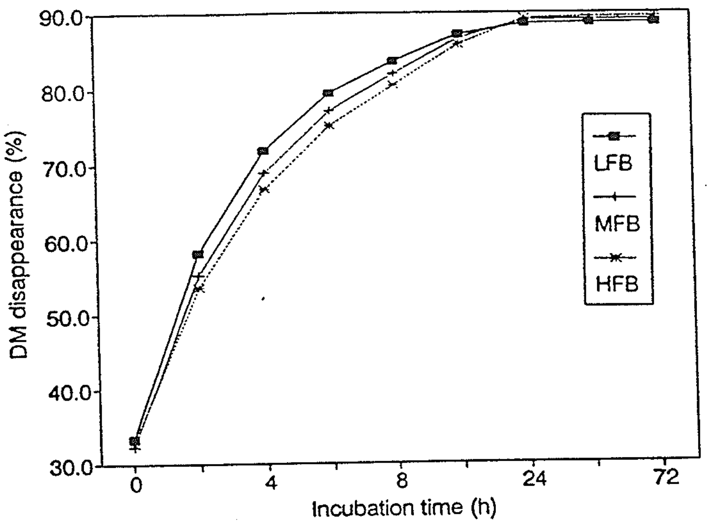
Source	DF	SS	MS	F value	Pr>F
Trt	2	0.00005	0.00003	1.34	0.331
Per(sq)	5	0.0003	0.00007	3.35	0.087
Cow	2	0.00002	0.000008	0.40	0.689
Sq*cow	2	0.00005	0.00003	1.40	0.318
Error	6	0.0001	0.00002		

Dependent variable: d

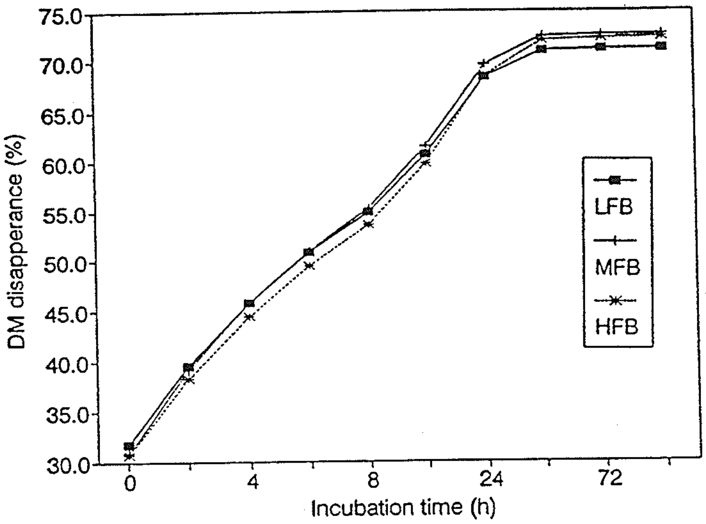
Source	DF	SS	MS	F value	Pr>F
Trt	2	0.17	0.08	0.13	0.881
Per(sq)	5	6.17	1.23	1.90	0.229
Cow	2	0.41	0.20	0.31	0.743
Sq*cow	2	2.56	1.28	1.97	0.220
Error	6	3.90			

Dependent variable: Effective NDF degradability

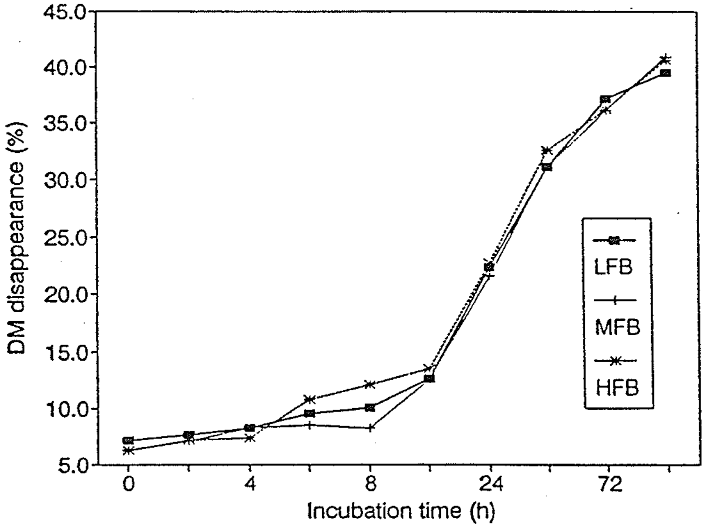
Source	DF	SS	MS	F value	Pr>F
Trt	2	1.62	0.81	1.72	0.257
Per(sq)	5	16.34	3.27	6.95	0.018
Cow	2	1.19	0.60	1.27	0.347
Sq*cow	2	1.89	0.95	2.01	0.215
Error	6	2.82	0.47		



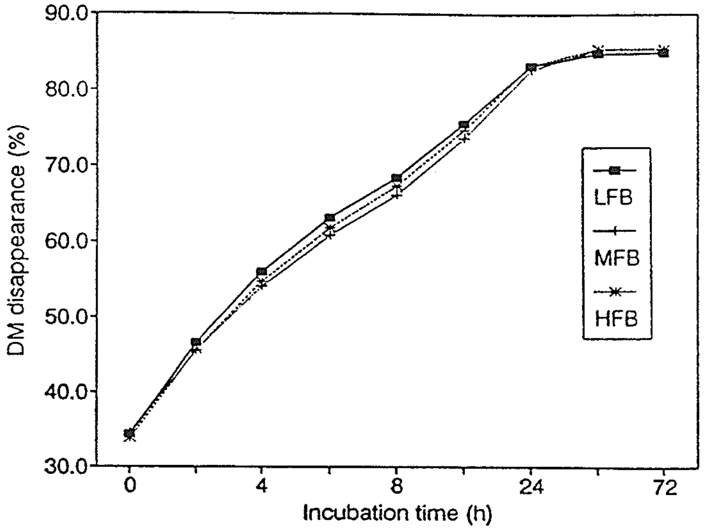
Effect of fungal biomass on DM disappearance in barley



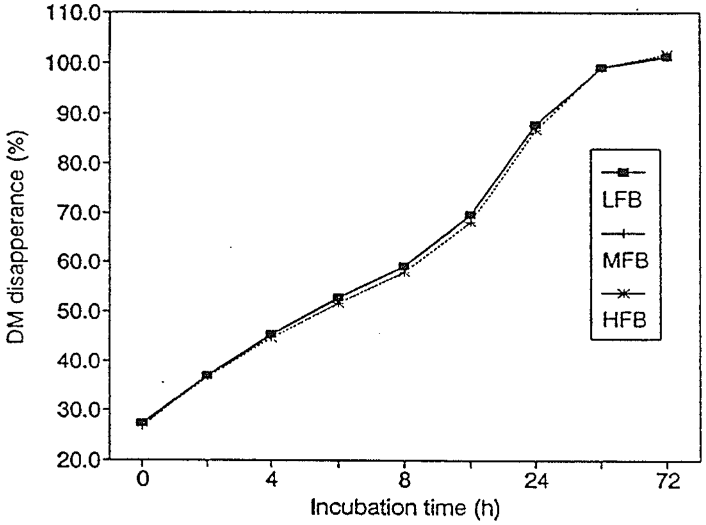
Dry matter disappearance of alfalfa hay



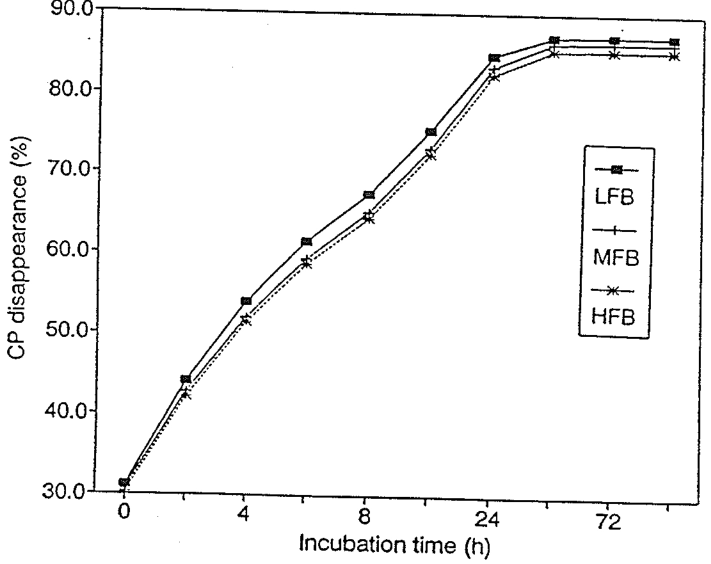
DM disappearance of barley straw



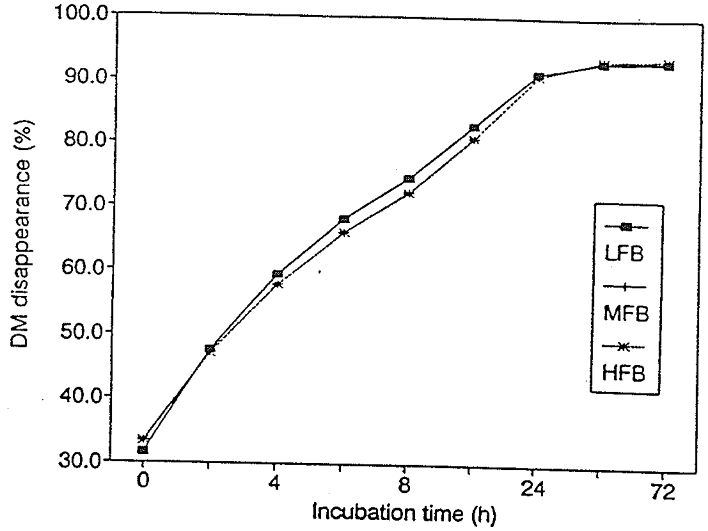
DM disappearance of canola meal



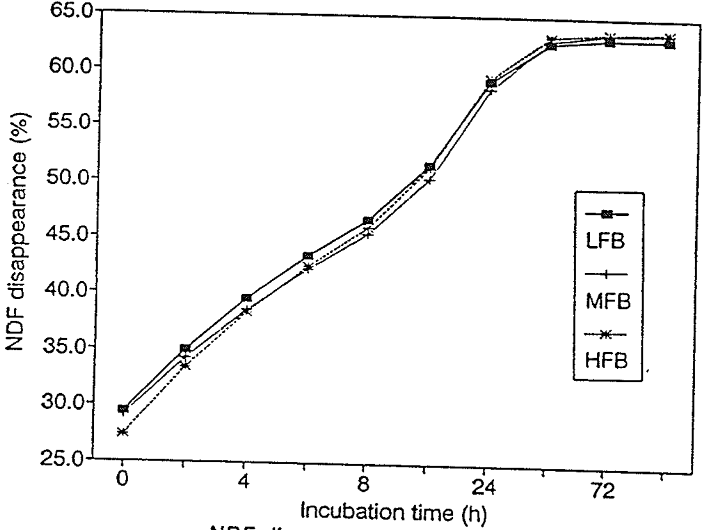
Effect of fungal biomass in hay on corn DM disappearance



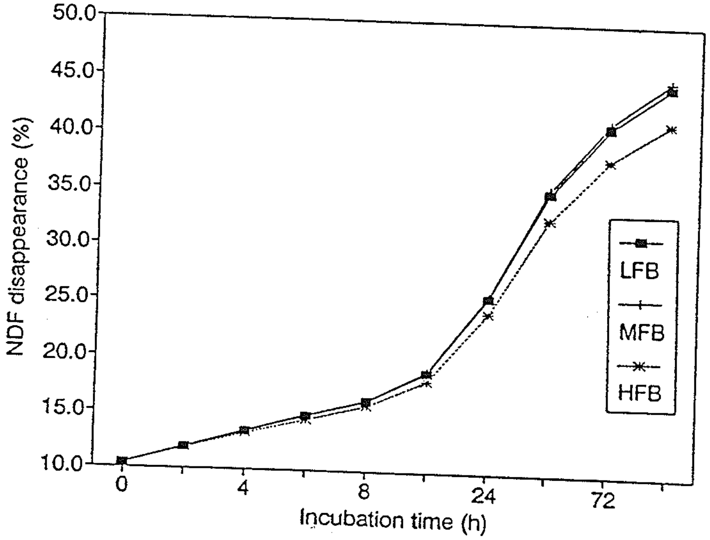
Disappearance of CP in alfalfa



CP disappearance of canola meal



NDF disappearance in alfalfa hay



NDF disappearance of barley straw