# GREEN FORAGES FOR DAIRY COWS

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“What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on.”

*- Jacques Yves Cousteau*

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# Preface

The present thesis entitled "Green forages for dairy cows" was submitted to the Graduate School of Science and Technology (GSST), Aarhus University, as a part of the requirements in the Ministerial Order on the PhD Programme at the Universities and Certain Higher Artistic Educational Institutions (PhD Order) to obtain the PhD degree. The work related to the PhD study was conducted between August 2014 and July 2017 at the Department of Animal Science, AU Foulum, Aarhus University.

The PhD study was financed by two research projects and GSST, with two third and one third, respectively. The two financing research projects were "Optimal mælkeproduktion med græs og bælgplanter" (English: Optimal milk production with grasses and legumes) funded by the Danish Milk Levy Fund and the Department of Animal Science, Aarhus University, and "Høsilage – optimering af proteinkvalitet til malkekøer" (English: Haysilage – optimisation of protein quality to dairy cows) funded by the Danish Milk Levy Fund and the Fund for Organic Farming. The first project was a cooperation between AU Foulum, SEGES and DLF, and the second project was a cooperation between AU Foulum and Organic Denmark.

The main objectives of the PhD project were to obtain knowledge on how green forages such as grasses and legumes affect feed intake and milk production in dairy cows and to obtain knowledge on how pre-wilting of grass-clover to a higher dry matter concentration before ensiling affects the protein value of the forage. An improved knowledge on these subjects can contribute to a combined optimisation of forage and milk production. Knowledge on the first subject was achieved through a meta-analysis and a production experiment with 36 dairy cows and knowledge on the second subject was obtained through an intensive experiment with four multi-fistulated dairy cows.

The thesis, which focuses on topics not described or discussed in the included papers, first introduces the overall issues and in the background (Chapter 2), characteristics and ensiling of green forage plants and their digestion by dairy cows are described before the hypotheses and aims are outlined in Chapter 3. Some of the key methods used in the thesis are discussed in Chapter 4 and Chapter 5 consists of six papers, which cover the results of the research conducted. Reliability and applicability of the obtained results are discussed in a broader context in Chapter 6. Finally, a general conclusion and future perspectives are presented.

Foulum, July 31st, 2017

Marianne Johansen

# Acknowledgement

First, I would like to thank my main supervisor Martin Riis Weisbjerg for giving me the opportunity to discover and examine this practice-oriented field of research, as forage quality and evaluation is. I find it very motivating, that results obtained from the research can be used directly by the agricultural advisory service and in the primary production. Furthermore, I want to thank Martin for sharing his huge knowledge, good discussions and for always taking the time to answer questions. I also want to thank my co-supervisors, Peter Lund and Karen Søegaard, for contributing with knowledge and ideas, and for reading and commenting manuscripts.

The experiments conducted during this PhD study had not been possible without engaged and skilled people in the barn to take care of the cows – a huge thanks to all of you for your effort! It was a pleasure to turn up in the barn. Furthermore, the sampling during the experiments had not been possible without help from Torkild Nyholm Jakobsen and Anne Louise Frydendahl Hellwing. Also the laboratory technicians in the "Animal Nutrition and Physiology"-group made a great effort in analysing all samples from the experiments. Thank you all.

During my PhD study, I had the pleasure to visit Dr Mary Beth Hall and her lab technician Jan Pitas at US Dairy Forage Research Center, Madison, USA, for eight weeks and I want to thank both of you for giving me the opportunity to work in your lab, good guidance and for making me feel welcome – it was a great experience. I also want to thank Jane Kay Rock for her kindness and hospitality – I am so glad that I could stay in your home.

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Finally, I want to give my deepest gratitude to Lasse Primdal for his love and support, for updating me on forage production in the "real" world, and especially for reminding me to look at things from a bird's eye view when everything seems chaotic and racing thoughts fill up the mind. Without you, I would not be where I am today.

# Contents

# Summary

High quality forages is required to meet the energy requirement of high yielding dairy cows. Cultivation of green forages is associated with environmental benefits compared to cultivation of maize, but there is a need of improved knowledge on feeding value of different green forages to optimise both forage cultivation and milk production.

The thesis comprises a meta-analysis and two experiments, and the results are presented and discussed in six papers. The meta-analysis included data from 43 published experiments and was used to compare feed intake and milk production in dairy cows fed different grass and legume species including perennial ryegrass, annual ryegrass, orchardgrass, timothy, meadow fescue, tall fescue, festulolium, white clover, red clover, lucerne and birdsfoot trefoil. In Experiment 1, 36 dairy cows were fed with perennial ryegrass, festulolium, tall fescue, red clover and white clover, which are the species most relevant for Danish conditions, and feed intake, milk production and eating behaviour was studied. During harvest of the crops, it was studied whether changes in leaf:stem ratio could be used to estimate field losses. In Experiment 2, four multi-fistulated, lactating cows were fed with grass-clover silages pre-wilted to dry matter (DM) concentrations ranging from 283-725 g/kg, and rumen metabolism and digestion of amino acids (AA) in the small intestine were studied. Changes in rumen protein degradation were compared with *in situ* measurements.

The meta-analysis and Experiment 1 showed that feed intake and milk production were higher in cows fed legume-based diets than in cows fed grass-based diets when forage organic matter (OM) digestibility was similar. Differences in milk production within different grass or clover species could be explained by differences in OM digestibility. The results indicated that there is an optimum for silage OM digestibility regarding milk production in the range 80-82%. The cows fed grass silage with a high OM digestibility (83.4%) did not produce the expected amount of milk based on the amount of OM actually digested in the gastrointestinal tract, and the feed intake when feeding white clover was probably regulated physiologically instead of physically. Experiment 1 also showed that changes in leaf:stem ratio can be used to estimate field losses if the plant material, collected in different steps of the harvesting process, is representative. Experiment 2 showed that the amount of AA digested in the small intestine increased with increasing silage DM concentration. The increase was caused by a reduced rumen degradation of feed protein, an increased rumen microbial synthesis and an increased small intestinal digestibility of AA. However, the AA profile of digested AA was negatively affected by increased silage DM concentration, as lysine and histidine in proportions of digested AA were reduced. The supply of all individual AA including those which might be first limiting was increased with increased silage DM concentration, and therefore, it will be beneficial to increase the DM concentration before ensiling. The results indicated that the observed effects could be considered as linear. The *in situ* technique seemed to be an adequate method to detect differences in rumen protein degradation.

The thesis indicates that there is room for improvement of OM digestibility in grass-clover silage in practice, that legumes should be included in the diet, and that grass-clover should be pre-wilted to 400-500 g DM/kg fresh matter before ensiling

# Sammendrag (Danish summary)

Grovfoder af høj kvalitet er nødvendig for at kunne dække højtydende malkekøers energibehov. Dyrkning af græsmarksafgrøder er forbundet med miljømæssige fordele sammenlignet med dyrkning af majs, men der er behov for mere viden om foderværdien af forskellige græsmarksafgrøder for at kunne optimere både grovfoderdyrkning og mælkeproduktion.

Afhandlingen omfatter en metaanalyse og to forsøg, og resultaterne er præsenteret og diskuteret i seks artikler. Metaanalysen inkluderede data fra 43 publicerede forsøg og blev anvendt til at sammenligne foderoptag og mælkeproduktion i malkekøer fodret med forskellige græs- og bælgplantearter, herunder almindelig rajgræs, italiensk rajgræs, hundegræs, timoté, engsvingel, strandsvingel, rajsvingel, hvidkløver, rødkløver, lucerne og kællingetand. I Forsøg 1 blev 36 malkekøer fodret med almindelig rajgræs, rajsvingel, strandsvingel, rødkløver og hvidkløver, som er de mest relevante arter under danske forhold, og foderoptag, mælkeproduktion og ædeadfærd blev undersøgt. Under høst af afgrøderne blev det undersøgt, om ændringer i stængel:blad forhold kunne bruges til at estimere marktab. I Forsøg 2 blev fire multi-fistulerede, lakterende køer fodret med kløvergræsensilage forvejret til tørstof(TS)-koncentrationer varierende fra 283-725 g/kg, og omsætningen i vommen og fordøjelsen af aminosyrer (AS) i tyndtarmen blev undersøgt. Ændringer i vomnedbrydning af protein blev sammenlignet med *in situ* målinger.

Metaanalysen og Forsøg 1 viste, at foderindtaget og mælkeproduktionen var højere i køer fodret med bælgplantebaserede rationer end i køer fodret med græsbaserede rationer når grovfoderets organisk stof (OS) fordøjelighed var sammenlignelig. Forskelle i mælkeproduktion indenfor forskellige græs- eller kløverarter kunne forklares med forskelle i OS fordøjelighed. Resultaterne indikerede, at der er et optimum for OS fordøjelighed i ensilage i forhold til mælkeproduktion i intervallet 80-82%. Køerne fodret med græsensilage med en høj OS fordøjelighed (83,4%) producerede ikke den forventede mængde mælk ud fra den mængde OS der faktisk blev fordøjet i mavetarmkanalen, og foderoptaget, når der blev fodret med hvidkløver, var sandsynligvis reguleret fysiologisk i stedet for fysisk. Forsøg 1 viste også, at ændringer i stængel:blad forhold kan bruges til at estimere marktab hvis plantematerialet, indsamlet i forskellige trin under høsten, er repræsentativt. Forsøg 2 viste, at mængden af AS fordøjet i tyndtarmen steg når ensilagens TS-koncentration blev øget. Stigningen skyldtes en reduceret vomnedbrydning af foderprotein, en øget mikrobiel syntese i vommen og en øget fordøjelighed af AS i tyndtarmen. Dog blev AS-profilen af fordøjet AS påvirket negativt af at øge ensilagens TS-koncentration, da lysin og histidin som andele af fordøjet AS faldt. Forsyningen af alle individuelle AS, også dem som kan være først begrænsende, steg med øget TS-koncentration i ensilagen, og det vil derfor være fordelagtigt at øge TS-koncentrationen inden ensilering. Resultaterne indikerede, at de observerede effekter kan betragtes som lineære. *In situ* teknikken var en tilfredsstillende metode til at detektere forskelle i vomnedbrydning af protein.

Afhandlingen indikerer, at der er plads til at forbedre OS fordøjelighed af kløvergræsensilage i praksis, at bælgplanter bør inkluderes i foderrationen og at kløvergræs bør forvejres til 400-500 g TS/kg frisk materiale før ensilering.

# List of included papers

I. **Johansen M.**, P. Lund and M.R. Weisbjerg.
*Feed intake and milk production in dairy cows fed different grass and legume spe-cies – a meta-analysis.*
Animal. Published online. DOI: 10.1017/S1751731117001215.

II. **Johansen, M.**, K. Søegaard, P. Lund and M.R. Weisbjerg.
*Digestibility and clover proportion determine milk production when silages of different grass and clover species are fed to dairy cows.*
Journal of Dairy Science. 2017, 100:8861-8880.

III. **Johansen M.**, A.L.F. Hellwing, P. Lund and M.R. Weisbjerg.
*Metabolisable protein supply to lactating dairy cows increased with increasing dry matter concentration in grass-clover silage.*
Animal Feed Science and Technology. 2017, 227: 95-106.

IV. **Johansen M.**, P. Lund and M.R. Weisbjerg.
*Amino acid profile of supplied metabolisable protein to lactating dairy cows is affected by dry matter concentration in grass-clover silage.*
Submitted.

V. **Johansen M.** and M.R. Weisbjerg.
*Comparison of protein degradation in the rumen measured in situ and in vivo.*
Grassland Science in Europe. 2016, 21: 213-215.

VI. **Johansen, M.**, K. Søegaard and M.R. Weisbjerg.
*Leaf:stem ratio as a tool to estimate field losses.*
Grassland Science in Europe. 2016, 21: 232-234.

# Abbreviations

| | |
|---|---|
| AA | amino acid(s) |
| ADF | acid detergent fibre |
| ADL | acid detergent lignin |
| CP | crude protein |
| $CH_4$ | methane |
| $CO_2$ | carbon dioxide |
| $Cr_2O_3$ | chromium oxide |
| DM | dry matter |
| DMI | dry matter intake |
| ECM | energy corrected milk |
| iNDF | indigestible neutral detergent fibre |
| MP | metabolisable protein |
| N | nitrogen |
| NPN | non-protein nitrogen |
| NDF | neutral detergent fibre |
| NIRS | near-infrared spectroscopy |
| NorFor | Nordic feed evaluation system |
| OM | organic matter |
| RUP | rumen undegraded feed protein |
| $TiO_2$ | titanium oxide |
| TMR | total mixed ration |
| VFA | volatile fatty acid(s) |
| $YbCl_3 \cdot 6H_2O$ | ytterbium chloride hexahydrate |

# 1 Introduction

In the last decades, the annual milk production per cow has increased in Denmark, and in 2015-2016 the recorded average annual energy corrected milk (ECM) yield was 10,453 kg/cow (RYK, 2016). The ongoing increase in milk yield is caused by genetic progress and by improved feeding and management (Ingvartsen *et al.*, 2003). Since dairy cows nowadays have a higher genetic potential for milk production, the demand for high quality feeds to meet the energy requirements increases.

Ruminants have developed the ability to utilise plant cell walls, as the ruminant digestive system consists of a large fermentation chamber where the feed is exposed to microbial fermentation. Monogastric animals cannot utilise plant cell walls, as mammalian digestive enzymes are not able to break down β-linked polysaccharides, which are found in cell walls. In high producing dairy cows, it is difficult to meet the energy requirements solely by forage with a high cell wall concentration, but the presence of cell walls in the diet is important for an optimal ruminal function and for animal health. However, when using high quality forages with high energy densities or high intake potentials, then forages can account for a large proportion in the diet (Jung and Allen, 1995). Simultaneously, concentrates cannot fully compensate for a low forage digestibility, thus highly digestible forage is important for a high production level independent of concentrate level (Kristensen and Skovborg, 1990; Randby *et al.*, 2012).

Maize silage and grass-clover silage are the most used forages for dairy cattle in Denmark (Kristensen *et al.*, 2015). However, maize silage has a lower crude protein (CP) concentration than grass-clover silage (Møller *et al.*, 2005), thus the need of purchased protein or other cultivated protein crops is higher, when the diet consists mainly of maize silage. Therefore, including more grass and especially legume silage in the diet will increase protein self-sufficiency. In addition, environmental benefits are associated with grass-clover production compared to maize production, as perennial forage crops protect against soil erosion and grass-clover production is associated with lower application of pesticides and fertiliser (Jung and Allen, 1995). Furthermore, the nitrogen (N) leaching per hectare from grass fields is only half the amount observed from maize fields (Kristensen *et al.*, 2008).

In the first part of the 20$^{th}$ century, red clover was an important crop in Danish agriculture due to its ability to fixate N, and in 1936, red clover was elected as the Danish national flower (Naturstyrelsen, n.d.). However, in the last part of the 20$^{th}$ century, widespread use of inorganic N fertiliser reduced the distribution of clover in the fields. In the last three decades the use of fertiliser has declined due to legal restrictions, thus focus on the benefits of having legumes in grass fields increases. However, scepticism about the feeding value of red clover to high producing dairy cows occurs in Danish milk production (Søegaard *et al.*, 2011). Besides the more widespread use of legumes, grass species as tall fescue and festulolium, which have some cultivation advantages compared to perennial ryegrass, have become more common in Danish agriculture in the last decade. However, little is known about the feeding value of these grass species. Therefore, more knowledge about the feeding value of different green forages as grasses and legumes is needed.

Even though green forage silages have a higher CP concentration than maize silages, it still can be difficult to fulfil the metabolisable protein (MP) requirement for high producing dairy cows, as a large part of the CP is degraded in the rumen (Tamminga *et al.*, 1991). The protein value of grass-clover silages is mainly affected by the distribution of true protein N and non-protein N (NPN), which is largely affected by the ensiling process. Therefore, the effect of increasing forage dry matter (DM) concentration before ensiling on the protein value was studied in the current thesis.

To optimise both forage and milk production, improved knowledge on feeding and protein value of green forages is needed. Therefore, the overall objectives were to evaluate feed intake and milk production in dairy cows offered different green forage species, and to measure the amount of MP supplied to dairy cows fed grass-clover silages pre-wilted to different DM concentrations before ensiling.

# 2 Background

This chapter outlines the characteristics of green forage plants, and describes in detail the anatomy and composition of plants and how the different fractions are digested in the cow, in order to explain the difference between grasses and legumes in their feeding value. The ensiling process, its effect on protein fractions and protein digestion by dairy cows are also described, but the first subject is weighted the most.

## 2.1 Green forage plants

Forage is defined as "edible parts of plants, other than separated grain, that can provide feed for animals, or that can be harvested for feeding" (Barnes *et al.*, 2007). In the current thesis, green forages are defined as perennial, cool-season, herbaceous crops cultivated in grass or pasture fields, which can be harvested or grazed several times during the growing season. Green forage plants belong to the botanical families *Poaceae* (grasses) and *Fabaceae* (legumes); however, only few species within these families belong to green forages as defined in this thesis.

### 2.1.1 Grasses

Grass seeds have only a single leaf when germinated and belong to the monocotyledons. All cool-season grasses are characterised by having the $C_3$ photosynthesis system. The root system of established grasses is adventitious and fibrous, and heavily branched in the upper soil layer. Most roots are within a soil depth of one meter (Moser and Jennings, 2007). Grass leaves consist of a free leaf blade and a leaf sheath that surrounds the stem, and are attached to the stem at nodes. Stems of vegetative tillers are short, whereas the internodes begin to elongate when there is a flowering stimulus. Only elongated stems are distinctly divided into nodes and internodes. Each plant consists of several tillers, and the productivity of a given grass species depends on tiller density and weight of individual tillers (Moser and Jennings, 2007).

A schematic representation of a generalised grass plant is shown in Figure 2.1 and an overview of characteristics of some common green forage grass species is given in Table 2.1. In north-western Europe, New Zealand and in other temperate regions of the world, perennial ryegrass is the most dominating green forage grass (Wilkins and Humphreys, 2003), as feeding quality and yield of perennial ryegrass is superior to other grasses when cultivated under ideal growing conditions (Casler and Kallenbach, 2007). However, perennial ryegrass has a low tolerance to drought, poor drainage, heat, low soil fertility or severe winters (Table 2.1) which often reduces the yield. Opposite, tall fescue has a very high drought tolerance because of its deep root system, but can also tolerate heat and poor drainage, and is well adapted to a wide range of environmental conditions. Feeding tall fescue has in the past been linked to severe disorders and reduced animal performance (Hemken *et al.*, 1984) because of the presence of the endophytic fungus *Neotyphodium coenophialum* that produces ergot alkaloids (Casler and Kallenbach, 2007) including ergovaline, which reduces milk produc-

tion (Kim *et al.*, 2007). However, endophyte-free cultivars and cultivars infected with endophytes not producing toxic alkaloids have been developed, and these cultivars do not negatively affect animal performance (Casler and Kallenbach, 2007). Timothy has a very high tolerance to severe winters and can survive low temperatures, by which it is the most sown grass species in Finland, Norway, Sweden, and in parts of Canada, where it can produce sufficient yields in spite of a short growing season (Wilkins and Humphreys, 2003). Other grass species can have other cultivation advantages and tolerate environmental challenges differently (Table 2.1). In the last decades, plant breeders have developed the new grass species festulolium (× *Festulolium*), which combines the feeding quality of *Lolium* species (annual ryegrass or perennial ryegrass) with the high persistency and drought tolerance of *Festuca* species (meadow fescue or tall fescue) (Thomas *et al.*, 2003). The attributes of festulolium depend on the parent species (Østrem *et al.*, 2013; Østrem *et al.*, 2015).

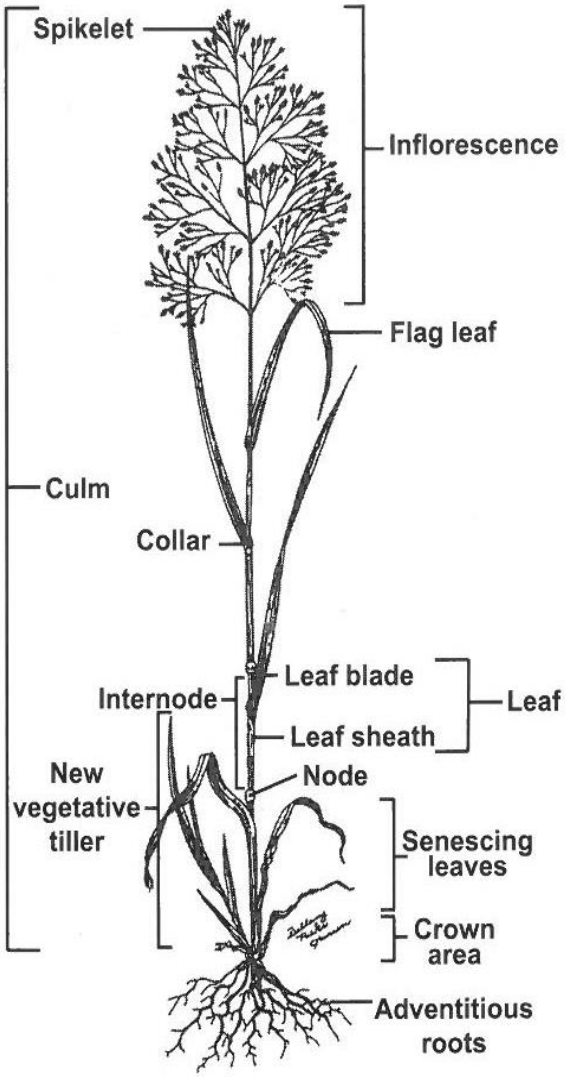


**Figure 2.1.** Schematic representation of a generalised grass plant showing a reproductive tiller with distinct nodes and internodes, a new vegetative tiller, the leaf parts and the adventitious root system (Moser and Jennings, 2007).

**Table 2.1.** Characteristics of some important grass species used as green forages. Compiled from Hall (1992); Casler and Kallenbach (2007) and Moore (2007).

| Common name | Perennial ryegrass | Annual ryegrass | Orchardgrass | Timothy | Meadow fescue | Tall fescue |
|---|---|---|---|---|---|---|
| **Scientific name** | *Lolium perenne* L. | *Lolium multiflorum* Lam. | *Dactylis glomerata* L. | *Phleum pratense* L. | *Festuca pratensis* Huds. | *Festuca arundinacea Schreb.* |
| **Origin** | Eurasia | Europe | Eurasia | Europe | ? | Europe, Africa |
| **Tolerance to**[1] | | | | | | |
| **low soil fertility** | ÷ | + | 0 | 0 | 0 | + |
| **drought** | ÷ | ÷ | 0 | ÷ | + | ++ |
| **poor drainage** | ÷ | + | + | 0 | 0 | ++ |
| **heat stress** | ÷ | ? | + | ÷ | 0 | + |
| **severe winters** | ÷ | ÷ | 0 | ++ | + | + |
| **frequent defoliation** | + | ? | + | ÷ | ? | + |

[1] ÷: low, 0: normal, +: high, ++: very high, ?: unknown

### 2.1.2 Legumes

Legume seeds have two embryonic leaves when geminated and therefore belong to the dicotyledons. Green forage legumes have a prominent taproot, which can be branched with smaller lateral roots (Moser and Jennings, 2007). Nodules, in which N-fixating rhizobia bacteria live, are present on the roots, and this symbiotic relationship supplies N to the plant. Therefore, input of N fertiliser is unnecessary when cultivated in pure stands, and input of N fertiliser can be reduced substantially when legumes are cultivated in mixes with grasses. Generally, legumes have a higher protein concentration than grasses (Albrecht and Beauchemin, 2003; Moser and Jennings, 2007). Normally, the leaves of vegetative legumes are compound and consist of three or more leaf blades, which are attached to a petiole that connects the leaf to the stem in an alternate order (Moser and Jennings, 2007).

A schematic representation of the most important green forage legumes is shown in Figure 2.2, and an overview of plant characteristics is given in Table 2.2. White clover differs from other forage legumes by its prostrate growing habit where stolons are growing along the soil surface, by which only leaves are harvested or grazed in the vegetative phase (Black *et al.*, 2009). The prostrate growing habit makes white clover able to scatter over a larger area, and shallow and adventitious roots are formed at nodes along the stolons. Birdsfoot

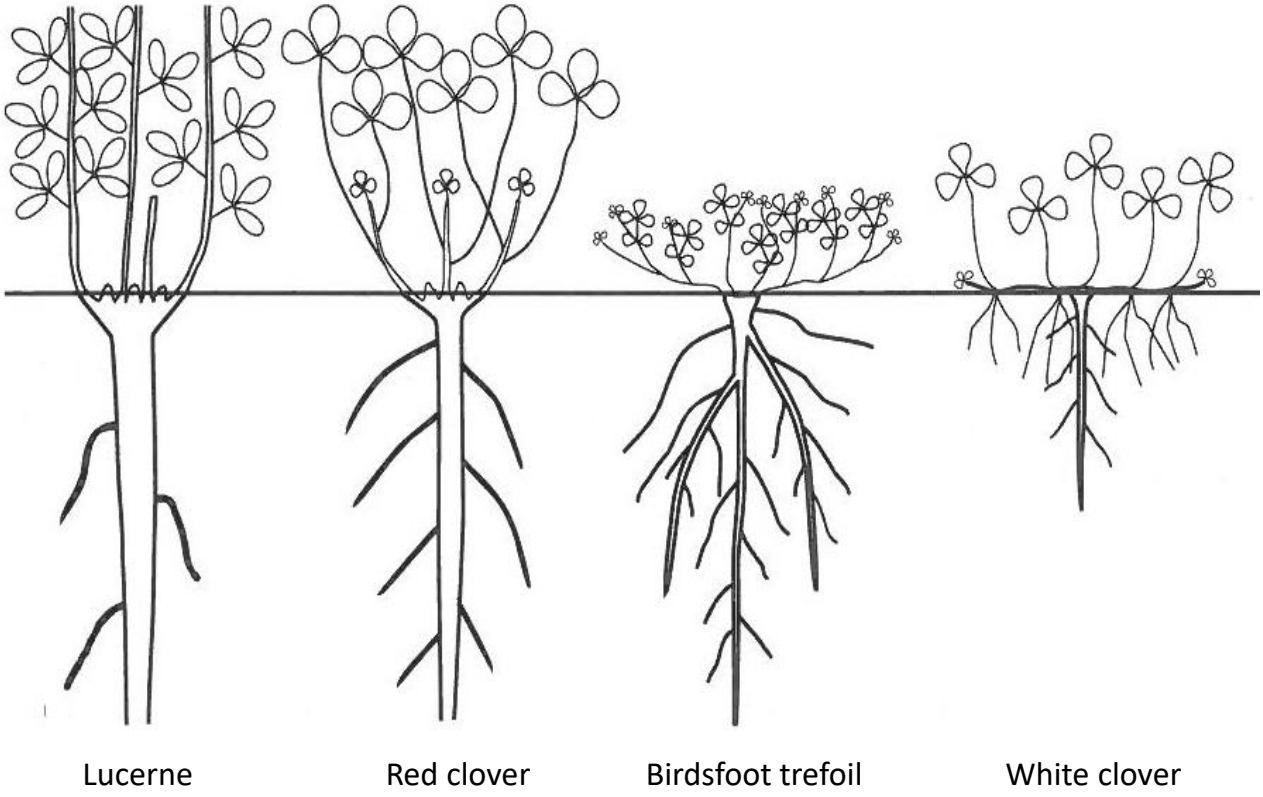


**Figure 2.2.** Schematic representation of the most important legume species used as green forages (McGraw and Nelson, 2007).

**Table 2.2.** Characteristics of the most important legume species used as green forages. Compiled from Moore (2007); Sheaffer and Evers (2007) and Hall (2008).

| Common name | Lucerne | Red clover | Birdsfoot trefoil | White clover |
|---|---|---|---|---|
| **Scientific name** | *Medicago sativa* L. | *Trifolium pratense* L. | *Lotus corniculatus* L. | *Trifolium repens* L. |
| **Origin** | Asia | Eurasia | Mediterranean | Eurasia |
| **Tolerance to**[1] | | | | |
| **low soil fertility** | ÷ | 0 | + | 0 |
| **soil acidity** | ÷ | 0/+ | + | 0/+ |
| **drought** | ++ | + | + | ÷ |
| **poor drainage** | ÷ | 0 | +/++ | +/++ |
| **frequent defoliation** | ÷/0 | 0/+ | +/++ | ++ |
| **Yield potential** | ++ | + | ÷/0 | 0 |
| **Growth habit** | Upright | Upright | Prostrate or upright | Prostrate |

[1] ÷: low, 0: normal, +: high, ++: very high

trefoil has an upright growing habit with fine and weak stems that can lodge, making the growth habit more prostrate, but not to the same extent as white clover (Sheaffer and Evers, 2007). Red clover and lucerne both have an upright growth with more rigid stems than white clover and birdsfoot trefoil, but vegetative stems of red clover remain relatively short compared to lucerne, making the stem proportion higher for lucerne than for red clover (Wilman

and Altimimi, 1984). The upright growth habit makes the yield potential higher for lucerne and red clover than for white clover and birdsfoot trefoil, however, the prostrate growth habit makes white clover and birdsfoot trefoil more tolerant to frequent defoliation. The tap-root of lucerne can reach a depth of eight meters, which makes lucerne extremely tolerant to drought (Moser and Jennings, 2007), whereas the more branched roots of white clover and birdsfoot trefoil make them more tolerant to poor drainage (Table 2.2). Lucerne is the least tolerant to unfavourable soil conditions, whereas birdsfoot trefoil is well adapted to several soil types as it has a high tolerance to soil acidity, low soil fertility, drought and poor drainage.

## 2.2 Anatomy and composition of plants

Living organisms are characterised by being composed of living cells, which primarily consist of carbon, hydrogen, oxygen, nitrogen, phosphorus and sulphur (Raven *et al.*, 2005). All eukaryotic cells consist of a plasma membrane, which encircles the protoplast in which the nucleus is located. Among other things, plant cells differ from animal cells by having a cell wall surrounding the plasma membrane, which has been an essential part of the evolution of terrestrial plants (Wilson, 1993). The cell wall contributes to strengthen the cell, and prevents the plasma membrane to rupture when the protoplast expands due to water uptake. Furthermore, size, shape and function of the cell are determined by the cell wall (Raven *et al.*, 2005). All plant cells have a primary cell wall, which is deposited before and during cell division and expansion, whereas only some plant cells have a secondary cell wall, which is deposited inside the primary wall when cell growth is completed (Wilson, 1993; Cosgrove, 2005).

### 2.2.1 The primary cell wall

The primary cell wall mainly consists of complex polysaccharides but also contains structural proteins and some phenolic compounds (McNeil *et al.*, 1984; Cosgrove, 2005; Scheller and Ulvskov, 2010). Polysaccharides in the cell wall, which are also called structural carbohydrates, are grouped into cellulose, hemicellulose and pectin. Schematic representations of polysaccharide structures are shown in Figure 2.3. Cellulose, which is the most well defined polysaccharide in the cell wall, is an unbranched β-4-linked D-glucan, in which the polymerisation can vary from hundred to several thousand glucose units (McNeil *et al.*, 1984; Scheller and Ulvskov, 2010). Cellulose chains form parallel strands called microfibrils, each containing several hundred cellulose chains, in which adjacent cellulose chains are cross-linked with hydrogen bonds (Raven *et al.*, 2005). Cellulose microfibrils make up the framework of the primary cell wall, are surrounded by other polysaccharides (Vogel, 2008) and constitute 15-30% of the dry mass (Table 2.3). Cellulose is insoluble in water and other organic solvents, but is dissolvable in strong mineral acid solutions e.g. 72% sulfuric acid (Giger-Reverdin, 1995).

Additionally, the primary cell wall consists of hemicellulose, which is a heterogeneous group of polysaccharides, characterised by having a backbone of β-4-linked glucose, xylose or mannose residues and being neither cellulose nor pectin (Scheller and Ulvskov, 2010).

The hemicelluloses are grouped into xyloglucans, xylans, mannans and glucomannans, and β-glucans (Scheller and Ulvskov, 2010). The type of hemicellulose present in the primary cell wall depends on plant type, species and cell type within plants (Vogel, 2008; Scheller and Ulvskov, 2010). In dicots, the most abundant hemicellulose is xyloglucan, whereas the concentration of xyloglucan in grasses is only minor (Table 2.3). The backbone in xyloglucans consists of β-4-linked D-glucose residues as in cellulose (Figure 2.3), but the glucan chain has xylosyl residues attached to three out of four glucosyl residues in a repeated structure, and some of the xylosyl residues furthermore have galactose and fucose residues added to the side chains (McNeil *et al.*, 1984; Cosgrove, 2005). The most abundant hemicelluloses in grasses are xylans, which are only found in minor amounts in dicots. Xylans are comprised of a backbone containing β-4-linked D-xylose residues to which different side chains are

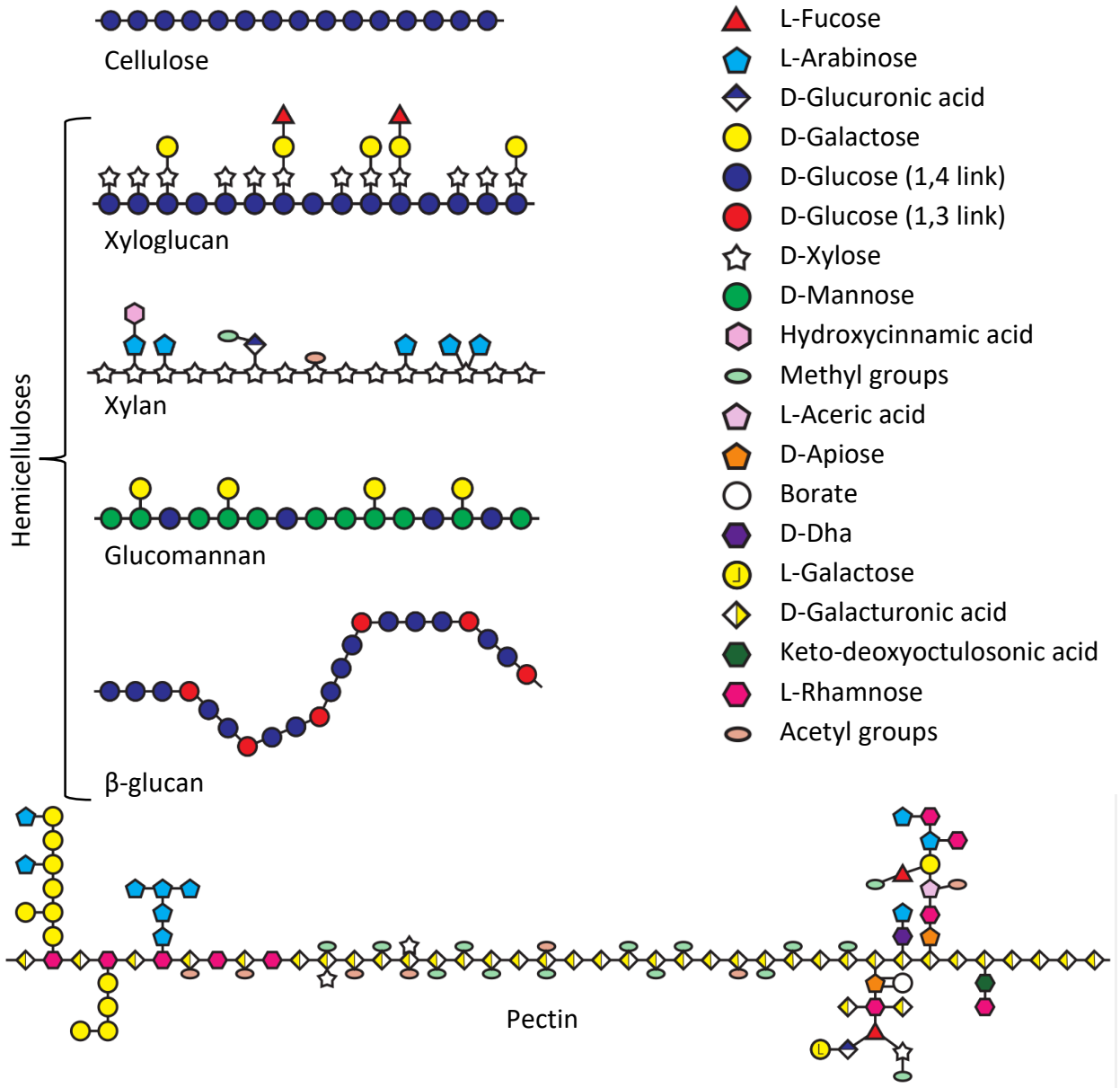


**Figure 2.3.** Structures of different cell wall polysaccharides in plants (Burton *et al.*, 2010).

**Table 2.3.** Generalised cell wall composition (% dry weight) of typical grass and dicot primary and secondary cell walls (Ishii, 1997; Vogel, 2008).

| | Primary wall | | Secondary wall | |
|---|---|---|---|---|
| | **Grass** | **Dicot** | **Grass** | **Dicot** |
| **Cellulose** | 20-30 | 15-30 | 35-45 | 45-50 |
| **Hemicelluloses** | | | | |
| **Xylans** | 20-40 | 5 | 40-50 | 20-30 |
| **β-Glucans** | 10-30 | Absent | Minor | Absent |
| **Xyloglucans** | 1-5 | 20-25 | Minor | Minor |
| **Mannans and glucomannans** | Minor | 5-10 | Minor | Minor |
| **Pectin** | 5 | 20-35 | 0.1 | 0.1 |
| **Lignin** | Minor | Minor | 7-15 | 20-30 |

attached (Figure 2.3). Arabinose residues are the most common branches (McNeil *et al.*, 1984), but glucuronic acid and ferulic acid ester residues can also be attached to the side chains (Cosgrove, 2005). Xylans can have different unrepeated structures, of which many variations are still not well known (Scheller and Ulvskov, 2010). Mannans, which have a backbone consisting entirely of β-4-linked mannose residues, and glucomannans, which have a backbone consisting of β-4-linked mannose and glucose residues in an unrepeated manner (Figure 2.3), are found in minor amounts in the primary cell wall of both grasses and dicots (Vogel, 2008; Scheller and Ulvskov, 2010). Both mannans and glucomannans can have galactose residues attached to their backbone (Vogel, 2008). The last group of hemicelluloses, the β-glucans, are only present in the cell wall of grasses (McNeil *et al.*, 1984; Vogel, 2008; Scheller and Ulvskov, 2010). The β-glucans consist of glucose residues, which are linked by both β-3- and β-4-linkages, that cause the chain to bend. Sequences of repeated β-4-linked glucose residues of variable length are separated by a single β-3-linkage (Figure 2.3). All β-glucans are unbranched (Vogel, 2008). As hemicelluloses are branched or have other modifications in their structure, the hemicelluloses are not able to form microfibrils as cellulose. However, xyloglucans, xylans, mannans and glucomannans can tightly bind to cellulose with hydrogen bonds and thereby bind cellulose microfibrils together, which strengthens and stabilises the cell wall (Cosgrove, 2005). These hydrogen bonds between the aforementioned hemicelluloses and cellulose require strong alkali to separate the hemicelluloses from the cell wall (McNeil *et al.*, 1984), whereas β-glucans can be easily extracted without acid (Scheller and Ulvskov, 2010).

The last group of polysaccharides, which is found in the primary cell wall, is pectin, a diverse group characterised by being rich in galacturonic acid residues (Willats *et al.*, 2001; Mohnen, 2008). In the primary cell wall of dicots, pectin can constitute up to 35% of the dry mass, whereas pectin only constitutes around 5% of the dry mass in grasses (Mohnen, 2008; Vogel, 2008). Pectin is most abundant in the middle lamella, which connect adjacent cells, and are found in minor amounts inside the cell wall (Raven *et al.*, 2005). Boiling water solubilises pectin, whereby pectin can easily be extracted (Giger-Reverdin, 1995). Together, the primary cell wall and the middle lamella is about 0.1-0.2 μm thick (Wilson, 1993).

### 2.2.2 The secondary cell wall

In some cells, a secondary cell wall is deposited when expansion of the primary wall is completed. Especially in cells involved in water conduction and structural strength, the secondary cell wall is important (Wilson, 1993). Cellulose microfibrils make up 35-50% of the dry mass in the secondary wall, whereas xylans and lignin make up the rest (Table 2.3). The remaining hemicelluloses found in the primary wall are nearly absent in the secondary cell wall (Vogel, 2008), and pectin substances are generally absent or found in low concentrations (Willats *et al.*, 2001). Often, three different layers can be distinguished in the secondary cell wall. The orientation of cellulose microfibrils differs in the three layers (Wilson, 1993; Raven *et al.*, 2005). Xylans in the secondary cell wall are less branched than xylans in the primary cell wall, which enables the xylans to bind more strongly to cellulose microfibrils, and thereby contributes to strengthen the cell wall (Vogel, 2008). The last component of the secondary cell wall is lignin (Figure 2.4), which is a complex, highly cross-linked, polyphenolic compound build-up of coumaryl, coniferyl and sinapyl alcohols (Jung and Deetz, 1993; Watkins *et al.*, 2015). The phenolic monomers of lignin can bind to hemicelluloses via hydrogen bonds or via ester linkages to the arabinose residues on xylans (Jung, 1989). Cell walls are lignified in varying degree depending on plant species, cell type and environmental factors (Buxton and Casler, 1993). Lignification starts in the middle lamella, proceeds through the primary cell wall and continues in the secondary cell wall as the cell ages. Therefore, the concentration of lignin is higher in the middle lamella and the primary wall than in the secondary wall. Normally, the thickness of the secondary cell wall is 1-3 µm (Wilson, 1993).

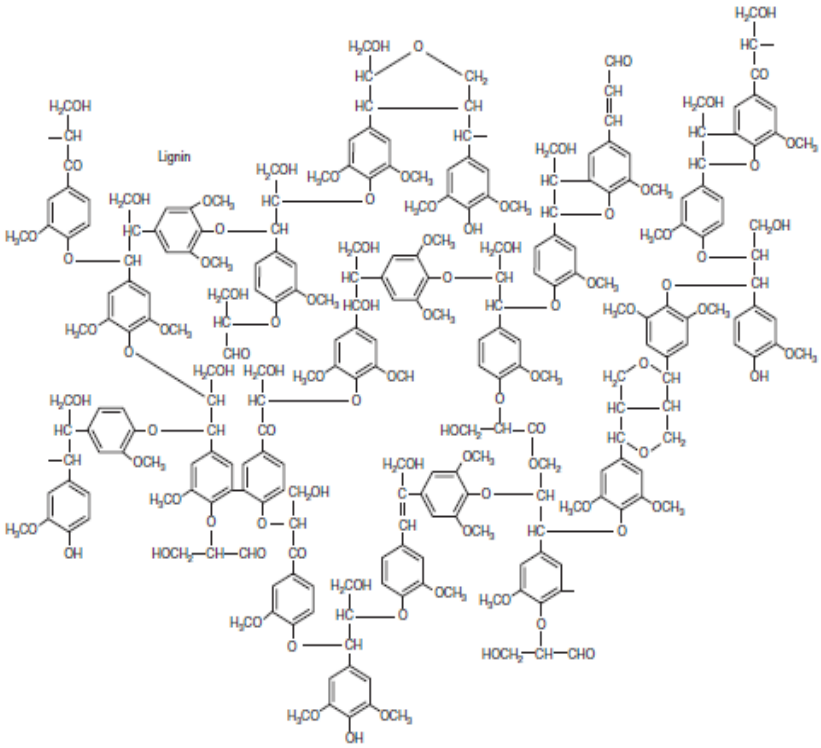


**Figure 2.4.** Schematic representation of the lignin structure, which are build-up of coumaryl, coniferyl and sinapyl alcohols (Watkins *et al.*, 2015).

### 2.2.3 Cell walls in ruminant feed evaluation

In feed evaluation systems for dairy cows, neutral detergent fibre (NDF) is used as a measure for cell wall constituents (Mertens, 2002; Volden, 2011a). The NDF method was originally developed by Van Soest (1963), but has been developed and updated and the procedure mostly used today is described by Mertens (2002). The NDF fraction consists of cellulose, some hemicelluloses and lignin as well as proteins associated with the cell wall. Pectin and β-glucans are not recovered in the NDF fraction, as they are soluble in boiling water and easily extracted from other cell wall components, as mentioned in section 2.2.1. Therefore, the NDF fraction describes the cell wall constituents that are insoluble in a neutral detergent, and not the biological plant cell wall structure. However, this is appropriate in ruminant feed evaluation, as pectin and β-glucans are easily fermented by microorganisms in the rumen (Jung and Allen, 1995). The cell wall constituents recovered in the NDF fraction are either slowly digested or totally indigestible (Mertens, 2002), and NDF is the chemical fraction which can differ the most in digestibility within and between plants (Wilson, 1994; Allen, 1996). This will be described further in section 2.4.1. The NDF fraction can be treated with an acid-detergent solution, which solubilises the remaining hemicelluloses, and the remaining fraction containing cellulose and lignin is called acid detergent fibre (ADF). Treatment of the ADF fraction with 72% sulfuric acid solubilises cellulose and the remaining fraction is called acid detergent lignin (ADL) (Van Soest, 1963).

### 2.2.4 Plant tissues

In plants, cells are grouped into three major tissue systems; dermal tissue, vascular tissue and ground tissue (Figure 2.5). The dermal tissue is the outermost protective layer, which is constituted by the epidermis of the primary plant body. Normally, the epidermis consists of a single layer of cells (Esau, 1960), in which the aerial located cell walls become thickened and lignified (Wilson, 1993). The aerial part of epidermal cells is also covered with a cuticle consisting of cutin and wax, which prevents water loss and microbial penetration and digestion (Wilson, 1993; Raven *et al.*, 2005). The vascular tissue is comprised of xylem, the primary water-conducting tissue, and phloem, the primary nutrient-conducting tissue, which enable transport of water, minerals and organic nutrients between roots and shoots (Esau, 1960). Commonly, xylem and phloem occur in strands called vascular bundles, which in leaves are called veins. The venation is parallel in grass leaves and netted in legume leaves (Esau, 1960). The phloem cells are thin-walled and do not lignify, whereas xylem cells are thick-walled with a high lignin concentration (Wilson, 1993). The ground tissue fills up the space between epidermis and vascular bundles, and is composed of parenchyma, collenchyma and sclerenchyma. Parenchyma, which is physiologically complex, makes up the largest part of the ground tissue and is found in the pith and cortex of the stem and in the mesophyll cells of leaves (Esau, 1960; Raven *et al.*, 2005). Mesophyll cells do not lignify and are loosely arranged in the leaves with 10-35% air space in grasses and 41-51% air space in legumes (Wilson, 1993). Most parenchyma cells only have a thin primary wall, and in legumes,

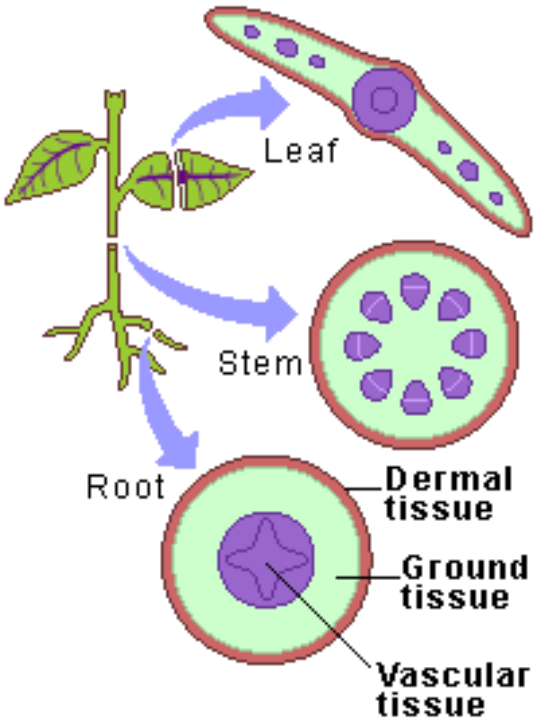


**Figure 2.5.** Illustration of location of the three major tissue systems within the plant body (Pearson, n.d.).

parenchyma cells do not develop a secondary, lignified cell wall. However, in grasses, parenchyma cells in stem and sheath can develop a thick lignified secondary cell wall (Wilson, 1993). Collenchyma cells, which are located beneath the epidermis in stems and below main veins in leaves, have soft and flexible thick primary walls and are adapted to support growth of leaves and stems (Esau, 1960; Raven *et al.*, 2005). Sclerenchyma tissue consists of long and narrow fibre cells that develop thick lignified secondary cell walls when mature, and the most important function of these cells is to provide tissues with hardness and rigidity (Esau, 1960). Fibre cells do only occur in small patches, with relatively thin-walled cells, around the main veins in leaves of legumes, whereas in grasses, thick-walled fibre cells surround vascular bundles in both blades, sheaths and stems (Wilson, 1993).

### 2.2.5 Plant growth and development

As plants grow and mature, the chemical composition of the whole plant changes since the cell wall fraction will make up a larger part of single cells, whereby the ratio between cell solubles and cell walls decreases (Buxton and Casler, 1993; Buxton and Redfearn, 1997). Therefore, the concentrations of cellulose, hemicellulose and lignin within the whole plant will increase with advancing maturity. However, within the cell wall fraction, the hemicellulose concentration will decrease with advancing maturity (Chen *et al.*, 2002), as cellulose and lignin make up a larger part of the secondary cell wall. The change in chemical composition with advancing maturity is also influenced by changes in leaf:stem ratio, as the stem will make up a larger proportion of the whole plant and stems have a higher cell wall concentration than leaves (Wilman and Altimimi, 1984; Wilson, 1994; Wilman and

Moghaddam, 1998). Due to the secondary wall thickening, old and more mature tissues have a higher lignin concentration than younger tissues, and furthermore, lignification is more pronounced in stems than in leaves as stems need more structural strength (Morrison, 1980; Wilson, 1993). Within the stem, the lignin concentration is higher in the basal internode than in the upper internode, but the difference in lignin concentration within the stem becomes smaller with advancing maturity (Wilson, 1993; Chen *et al.*, 2002).

Maturity and chemical composition of green forage plants are not only affected by plant age, but are also influenced by environmental factors, where temperature, solar radiation, water deficiency and nutrient availability are the most important (Buxton, 1996). Temperate forage species achieve optimal growth at temperatures around 20°C. Therefore, increasing temperature will increase plant development. Higher concentrations of sugar and water-soluble carbohydrates occur when temperature is below the optimum for growth, as photosynthesis is not as sensitive as growth to lower temperatures (Buxton, 1996) and an increasing temperature stimulates the conversion of soluble carbohydrates into structural carbohydrates (Van Soest *et al.*, 1978). Furthermore, sugar and water-soluble carbohydrates are accumulated in the leaves during the photoperiod where plants are exposed to light due to photosynthesis, giving rise to diurnal and seasonal variations in the concentration of these (Buxton, 1996). Besides stimulating growth, higher temperatures also increase lignification (Buxton and Casler, 1993; Wilson, 1994), therefore plants grown at high temperatures will have a higher lignin concentration than plants grown at lower temperatures, even at a similar maturity stage (Buxton, 1996). The effect of temperature on lignification is more pronounced in grasses than in legumes (Wilson, 1994).

Water deficiency reduces growth and plant development, by which the maturity at a given age will be less (Buxton, 1996). Therefore, plants exposed to moderate water stress will have a lower cell wall concentration (Wilson, 1994). Opposite, if plants are exposed to more severe water stress, senescence of older leaves is accelerated as proteins and water-soluble carbohydrates are translocated to the roots, by which the leaf mass is reduced resulting in a lower leaf:stem ratio (Buxton, 1996). However, water stress does influence biomass yield more than chemical composition (Buxton and Casler, 1993).

### 2.2.6 Protein

In green forage plants, 75-90% of the total N is present in true protein, which is a complex organic compound build-up of amino acids (AA). The remaining is present as NPN such as free AA, amides, amines, nucleotides, chlorophyll and nitrate (Ohshima and McDonald, 1978). The protein concentration decreases with advancing maturity (Buxton and Marten, 1989) due to a decreasing leaf and stem protein concentration, but also because of the changes in leaf:stem ratio (Buxton, 1996). Enzymes make up the majority of proteins in plant tissues and about half of the enzyme material is ribulose bisphosphate carboxylase-oxygenase (Whitehead, 1995), a photosynthetic enzyme. Leaf tissue is specialised in photosynthesis, thus many chloroplasts, which are the organelles in which the photosynthesis is conducted, are present in the leaves (Esau, 1960). Therefore, more protein is located in the leaf blade than in the stem (Mowat *et al.*, 1965; Alli *et al.*, 1985). The AA profile of leaf protein

does not vary much between plant species (Carpintero *et al.*, 1979). The protein concentration in green forages is highly dependent on the N availability. In grasses, N fertilisation is important to increase the protein concentration and DM yield, whereas in legumes, N is made available via the N-fixating bacteria, thus making the protein concentration higher in legumes than in grasses (Buxton, 1996).

## 2.3 Ensiling and storage

In areas with periods of little or no forage growth, such as winters in temperate areas, preservation as silage or hay making is important to supply dairy cows with energy, protein and fibre during a period, where fresh forage is unavailable (Rotz and Muck, 1994; Wilkinson *et al.*, 2003). In temperate areas including Denmark, making silage is preferred to hay, as the process is less weather dependent (Pahlow *et al.*, 2003). Additionally, harvest of green forages at optimal developmental stages several times during the growing season makes silage production the best way to maximise and preserve nutritional value of a given crop for ruminants (Pahlow *et al.*, 2003). Therefore, many farmers use silage for feeding dairy cows all year round. However, the nutritional value of a stored forage is lower than the fresh forage prior to harvest, because of nutrient changes and losses during the preservation process (Moser, 1980).

In Denmark, normal practice is to pre-wilt the forage on swaths after mowing to increase DM concentration to 300-350 g/kg which is the level that substantially reduces effluent losses and ensures good fermentation (Muck *et al.*, 2003). After mowing, plant enzymes remain active and the respiration process causes losses of water-soluble carbohydrates (Moser, 1980). Higher temperatures increase the magnitude of respiration, which continues until inhibited by anaerobic conditions, high DM concentrations (above 700 g/kg) or by lack of substrates for respiration (McGechan, 1989). Furthermore, plant proteases cause proteolysis, where plant proteins are hydrolysed to peptides, free AA and amides (Muck *et al.*, 2003). However, polyphenol oxidases, which are present in some plants e.g. red clover, can deactivate plant proteases and thereby reduce proteolysis (Lee, 2014). Proteolysis by plant enzymes occurs both during wilting and during the ensiling process, but the rate of proteolysis is reduced significantly by increased DM concentration (Muck, 1987; Slottner and Bertilsson, 2006), and by reduction of silage pH (Rotz and Muck, 1994). Rapid wilting to the desired DM concentration is necessary to reduce proteolysis and respiratory losses. During wilting, plant material synthesises proline (Kemble and MacPherson, 1954) as a response to the osmotic stress, since proline acts as an osmolyte (Delauney and Verma, 1993). Besides respiratory losses, losses in the field can be caused by leaching of especially water-soluble carbohydrates due to rain, or by losses of fragments of plant material due to mechanical operations (McGechan, 1989). The drying rate of leaf material is faster than the drying rate of stem material (Menzies and O'Callaghan, 1971; Alli *et al.*, 1985), and as the different parts dry they become more susceptible to be lost (McGechan, 1989). Therefore, there is an increased risk of losing leaf material compared to stem material.

### 2.3.1 The ensiling process

Establishment and retention of an anaerobic environment and the formation of lactic acid are the two most important parameters for a successful ensiling of forage crops (Muck, 2010). For a quick establishment of an anaerobic environment, the forage has to be well-compacted, which is easier if the forage is finely chopped, and sealed immediately (McDonald *et al.*, 1991). When the anaerobic environment is established, lactic acid bacteria will ferment water-soluble carbohydrates to mainly lactic acid, which reduces the pH (Figure 2.6a). Acetic acid, carbon dioxide ($CO_2$) and ethanol will be produced in minor amounts depending on the lactic acid bacteria species present. The drop in pH is important to inhibit the growth of undesirable microorganisms as enterobacteria and clostridia (Muck, 2010). The enterobacteria are competitors to the lactic acid bacteria regarding the water-soluble carbohydrates in the beginning of the ensiling process, where pH is optimal for their growth (McDonald *et al.*, 2011). The primary fermentation product from enterobacteria is acetic acid, but succinate, butanol, ethanol and $CO_2$ are also produced, whereby their fermentation is less desirable than that of lactic acid bacteria (Muck, 2010). Enterobacteria also decarboxylate and deaminate AA, whereby ammonia is produced (Fijalkowska *et al.*, 2015). Clostridia enter the silage as spores originating from soil contamination, and their activity occurs later in the ensiling process (McDonald *et al.*, 2011). Saccharolytic clostridia ferment the remaining water-soluble carbohydrates and lactic acid to mainly butyric acid, whereas proteolytic clostridia ferment AA to various compounds such as ammonia, amines, butyric acid and $CO_2$ (Muck, 2010). Clostridia are not wanted in silage as feed intake by dairy cows is reduced, the silage become unstable, and major losses of energy occur (McDonald *et al.*, 1991).

The drop in pH depends on the amount of water-soluble carbohydrates available for fermentation by the lactic acid bacteria and the forage DM concentration. An increased DM concentration reduces microbial activity because of an increased osmotic pressure (McDonald *et al.*, 1991). This does also increase the critical pH value by which the silage can be considered anaerobically stable, which is the pH value that inhibits the growth of clostridia (Pahlow *et al.*, 2003). If the amount of water-soluble carbohydrates is insufficient for

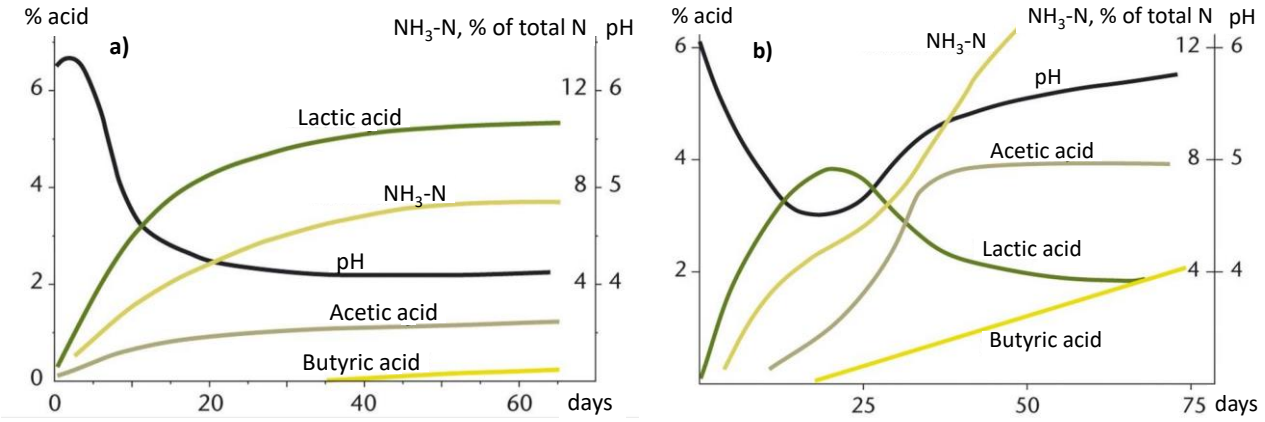


**Figure 2.6.** Development in pH and concentration of fermentation acids and ammonia N ($NH_3$-N) during a successful ensiling process (**a**) and an ensiling process with secondary fermentation by clostridia (**b**) (Nielsen *et al.*, 2003).

the lactic acid bacteria to reduce pH to the critical value, secondary fermentation by clostridia can occur as illustrated in Figure 2.6b. The amount of water-soluble carbohydrates needed and the speed of reducing pH depends on the buffer capacity of the forage, which is affected by organic acids, phosphates, sulphates, nitrates, chlorides and proteins (O'Kiely and Muck, 1998). Usually, the buffer capacity is higher in legumes than in grasses (McDonald and Henderson, 1962).

## 2.4 Digestive system of cows

The gastrointestinal tract of the cow (Figure 2.7) consists of the mouth, oesophagus, a complex four-compartment stomach including the rumen, reticulum, omasum and abomasum, the small intestine and the large intestine, which together with the caecum is called the hindgut. The chewing activity in the mouth ensures an efficient comminution of the feed during eating and especially during rumination, where feed boluses from the rumen are returned to the mouth for further mastication. In the mouth, the feed is also diluted with saliva which contains bicarbonate and phosphate buffers, which help to maintain the pH in the rumen at 5.5-6.5 (McDonald *et al.*, 2011). Depending on feed characteristics, such as NDF concentration and particle size, and animal factors, the saliva production in dairy cows can vary from 100-250 L/day (Meyer *et al.*, 1964; Maekawa *et al.*, 2002b, 2002a). The first two compartments of the stomach, the rumen and the reticulum, are not separate compartments, and constitute the reticulo-rumen. The reticulo-rumen is a 100-150 L anaerobic fermentation chamber colonised by bacteria, archaea, protozoa and fungi, which help to digest the feed (McDonald *et al.*, 2011). After exposure to microbial digestion in the reticulo-rumen, the digesta passes through the omasum, where water and other components are absorbed, and further into the abomasum, where enzymes and hydrochloric acid are secreted

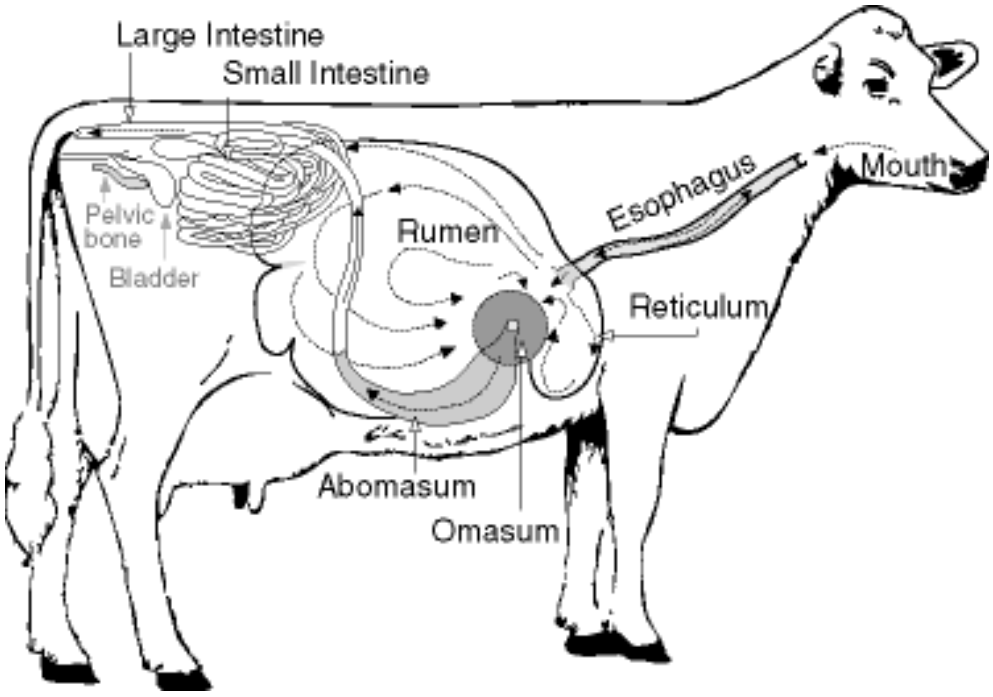


**Figure 2.7.** Schematic representation of the gastrointestinal tract of the cow (Hyun-June, 2014).

(Forbes and France, 1993). In the first part of the small intestine, the duodenum, digesta is mixed with bile secreted from the liver and digestive enzymes secreted from the pancreas. Digestion and absorption of nutrients takes place during the passage through the two additional segments of the small intestine, the jejunum and ileum. In the hindgut, the digesta is again exposed to microbial fermentation and volatile fatty acids (VFA) and water are absorbed before the digesta is excreted as faeces (Forbes and France, 1993).

### 2.4.1 Digestion of carbohydrates

A large part of the carbohydrates in feed are metabolised by microbes in the rumen. The microorganisms attach to the feed particles and extracellular microbial enzymes break down the polysaccharides into simple sugars, which immediately are taken up by the microorganisms and metabolised intracellularly (McDonald *et al.*, 2011). The major end products of carbohydrate digestion in the rumen are acetate, propionate, butyrate, $CO_2$ and methane ($CH_4$). Acetate, propionate, butyrate and other VFA provide energy to the cow (between 50-70% of the digestible energy intake) and about 80-90% of the VFA are absorbed across the rumen wall, while the remaining is absorbed in the omasum, abomasum and small intestine (France and Siddons, 1993; McDonald *et al.*, 2011). The VFA profile is dependent on the type of carbohydrates fermented, as forage diets high in NDF increase the production of acetate, whereas diets high in starch enhance the production of propionate (France and Siddons, 1993). The difference in VFA profiles between different diets is due to the competition between rumen microorganisms which is affected by the gain of energy from the available substrate, the hydrogen pressure in the rumen influenced by the balancing of redox reactions, and pH (Kristensen *et al.*, 2003). The produced $CO_2$ and $CH_4$ are lost by eructation and therefore, provide no energy for the cow (McDonald *et al.*, 2011).

The digestibility of different carbohydrates in the rumen is dependent on the rate of microbial digestion and the passage rate out of the rumen (Allen and Mertens, 1988). For the NDF fraction, the digestion rate relative to the passage rate is slow, whereas the digestion rate of cell solubles, such as sugars and starch, and water-soluble cell wall constituents, such as pectin and β-glucans, is fast. Typical rates of digestion are 300-700%/h for sugars (Weisbjerg *et al.*, 1998), 20-40%/h for starch (Tothi *et al.*, 2003) and pectin (Hatfield and Weimer, 1995), and 2-7%/h for NDF (Weisbjerg *et al.*, 2003). Therefore, the rumen digestibility of cell solubles and water-soluble cell wall constituents is high, and only a minor part is normally escaping the rumen and digested in the small intestine or in the hindgut (Huhtanen *et al.*, 2006). Many factors influence the digestion rate of the NDF fraction. Cellulolytic bacteria have to attach to the surface to digest cell wall polysaccharides, thus the area for colonisation relative to the volume of the cell wall affects the digestion rate (Wilson, 1994). In cells with only a thin primary wall e.g. mesophyll cells, the ratio between the surface area and the cell wall volume is high, and the degradation rate for these cell walls is fast (Wilson, 1993; Wilson and Kennedy, 1996). In cells with a thick secondary cell wall as in xylem and sclerenchyma, the ratio between surface area and cell wall volume is low. Furthermore, the microbes have no access to the outer cell wall surface as the middle lamella has a high lignin concentration and thus is indigestible (Wilson and Hatfield, 1997). Therefore, the cells have to be physically cracked, to ensure that microbes can get access to the cell

lumen. However, the lumen is narrow and only a small surface area is available for microbial colonisation, thus the digestion rate of cell walls in these cell types is slow (Wilson, 1993).

Besides cell wall thickness, microbial access to the cell wall polysaccharides is also affected by lignin. Rumen microorganisms are not able to metabolise lignin, and lignin acts as a physical barrier hindering the microbial access to cellulose and hemicellulose (Buxton and Redfearn, 1997). The lignin concentration in the cell wall thus affects the NDF degradation rate. In general, the slow NDF degradation rate is not due to cellulose and hemicellulose being difficult to degrade as such, but merely because of the restricted access to the polysaccharides, especially in cell walls with secondary wall thickening. Therefore, an effective comminution of feed particles into small particles is important to obtain an effective microbial degradation and to maximise the energy gained from cell walls (Wilson, 1994).

In addition to microbial access to cell wall polysaccharides, the rumen environment also affects the digestion rate of NDF. If the supply of nutrients e.g. N to the microbes is insufficient, the microbial fermentation is impaired (Mehrez *et al.*, 1977; Wilson and Kennedy, 1996). Furthermore, cellulolytic bacteria are sensitive to low rumen pH, and at a pH below approximately 6.2, the NDF degradation rate is reduced markedly (Mould *et al.*, 1983; Huhtanen *et al.*, 2006). Also unsaturated and medium chain fatty acids have a toxic effect on rumen bacteria, thus fat and fatty acid supplementation to a diet can reduce NDF degradation (Huhtanen *et al.*, 2006). Moreover, some cellulolytic bacteria are able to degrade starch and as the preference is higher for the easily fermentable carbohydrates, the rate of NDF degradation is reduced with an increased starch content in the feed (Mould *et al.*, 1983; Weisbjerg *et al.*, 2003).

As rumen digestibility of NDF is depended on the competition between degradation and passage, an increased passage rate will reduce NDF digestibility in the rumen (Allen and Mertens, 1988). Normally, the passage rate increases with increased feed intake (Okine and Mathison, 1991; Huhtanen *et al.*, 2006). However, the passage of particles out of the rumen is not random, as newly ingested, digestible and large particles are selectively retained in the rumen, whereas small and aged particles with a low concentration of digestible material are allowed to pass out of the rumen (Figure 2.8) (Allen and Mertens, 1988; Huhtanen *et al.*, 2006). Physical distention of the reticulo-rumen is a major factor affecting intake of forages (Mertens, 1994), as the rumen has a limited capacity and particles are retained in the rumen. In diets, where particles are retained in the non-escapable pool for a long time, the intake is reduced compared to diets, where particles are released faster to the escapable pool. Because of the selective retention of feed particles in the rumen, most of the NDF is degraded in the rumen, whereas less than 5% of the total NDF digestion takes place in the hindgut (Huhtanen et al., 2006).

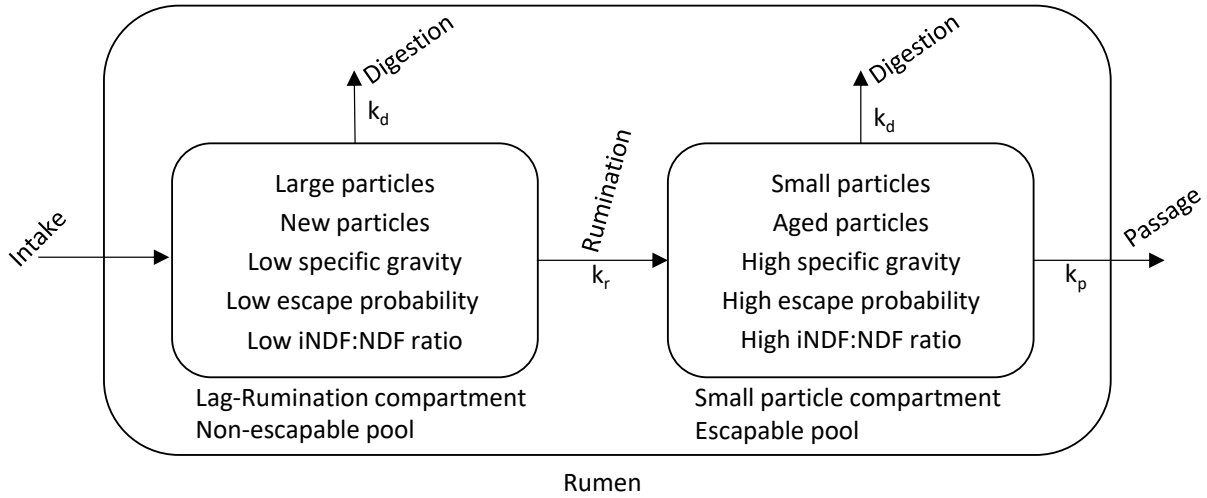


**Figure 2.8.** Schematic representation of the selective retention of feed particles in the rumen. Particles in the non-escapable pool are either digested or released to the escapable pool, as the particles have a low probability of escaping the rumen. The rate of release ($k_r$) from the non-escapable to the escapable pool depends on particle size breakdown influenced by rumination and changes in specific gravity and the iNDF:NDF ratio caused by digestion influenced by the rate of digestion ($k_d$). Particles in the escapable pool can be digested or pass out of the rumen with the rate of passage ($k_p$). Modified from Allen and Mertens (1988) and Huhtanen et al. (2006).

### 2.4.2 Digestion of protein

Crude protein (CP) in feed is defined as N × 6.25 and consists of both true protein and NPN. The true protein can be hydrolysed by microbial proteases in the rumen to peptides and AA or pass through the rumen as rumen undegraded feed protein (RUP) as illustrated in Figure 2.9 (McDonald *et al.*, 2011). The AA are either utilised by the microorganisms in the rumen to synthesise microbial protein or deaminated. Ammonia is formed by the deamination and the remaining carbon-skeleton is metabolised to VFA and $CO_2$ (Walker *et al.*, 2005). A variety of VFA including branched-chain VFA is produced by ruminal AA degradation (El-Shazly, 1952). Most NPN is readily degraded when entering the rumen and the N of this component will enter the ammonia pool. Microorganisms in the rumen can utilise the ammonia to synthesise microbial protein. However, the efficiency of microbial protein synthesis depends not only on the amount of N available, but also on the availability of energy (Clark *et al.*, 1992). Excess ammonia is absorbed through the rumen wall and transferred via the blood to the liver, where it is converted to urea. Via saliva or through the rumen wall, urea can be recycled to the rumen. Excess urea is excreted in urine or milk and thus wasted (McDonald *et al.*, 2011). A high proportion of N will be excreted in urine or milk if protein is rapidly digested in the rumen and microbial synthesis is restricted due to a lack of available energy (Buxton, 1996). Proteins can be protected from microbial breakdown by some plant metabolites e.g. tannins (Makkar, 2003) and polyphenol oxidases (Lee, 2014) which will increase the amount of RUP.

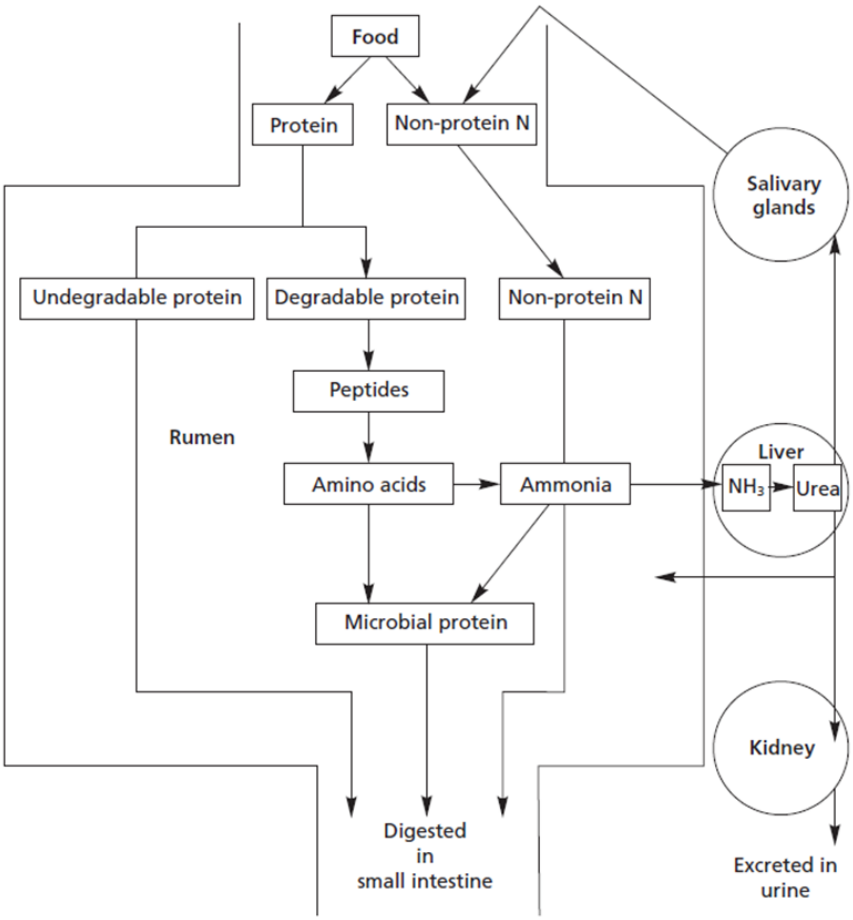


**Figure 2.9.** Schematic representation of the metabolism of nitrogenous compounds in the rumen (McDonald *et al.*, 2011).

Microbial protein and RUP that escape the rumen will be hydrolysed to peptides by pepsin in the acid environment of the abomasum. In the small intestine, digestive enzymes cleave the peptides into AA, which are absorbed by the cow (McDonald *et al.*, 2011). Besides RUP and microbial protein synthesised in the rumen, AA available for absorption also derives from endogenous protein (Clark *et al.*, 1992). AA digested and absorbed in the small intestine is defined as MP. Additional microbial protein synthesis occurs in the hindgut, but the microbes synthesised in the hindgut are excreted in faeces and their nutritional value is lost (Walker *et al.*, 2005).

## 2.5 Green forages in dairy cow nutrition

The energy concentration of green forages is reflected in the digestibility of organic matter (OM), which is a measure of the overall quality of forages (Allen, 1996). As described in section 2.4.1, NDF is degraded slowly in the rumen and therefore, is the fraction that can differ the most in digestibility, whereas the digestibility of other nutrients is often high and constant. Therefore, NDF concentration and NDF digestibility are the variables that affect OM digestibility most.

**Table 2.4.** Variation in chemical composition, yield and stem fraction for the spring growth and the second regrowth of perennial ryegrass, festulolium, white clover, red clover and lucerne harvested on AU Foulum in 2006. Three harvest dates with one week intervals are behind the range for each growth. The values are derived from Weisbjerg *et al.* (2010).

| | CP (% of DM) | NDF (% of DM) | ADL (% of DM) | Yield (hkg DM/ha) | Stem fraction (%) |
|---|---|---|---|---|---|
| **Perennial ryegrass** | | | | | |
| **Spring growth** | 24.5-14.4 | 38.8-45.2 | 1.37-1.74 | 17.3-39.2 | 25-53 |
| **Second regrowth** | 17.8-14.6 | 52.2-59.9 | 2.44-3.48 | 14.9-21-5 | 5-21 |
| **Festulolium** | | | | | |
| **Spring growth** | 21.4-13.6 | 35.7-46.3 | 1.15-1.78 | 28.6-50.1 | 36-63 |
| **Second regrowth** | 15.2-11.8 | 55.7-60.2 | 3.44-3.83 | 20.0-29.2 | 52-67 |
| **White clover** | | | | | |
| **Spring growth** | 30.1-27.7 | 17.6-19.7 | 2.01-2.42 | 9.7-21.7 | 0 |
| **Second regrowth** | 23.3-25.5 | 32.4-34.2 | 7.63-7.09 | 24.5-19.0 | 47-31 |
| **Red clover** | | | | | |
| **Spring growth** | 27.7-21.2 | 22.4-29.9 | 1.77-2.51 | 19.6-40.3 | 3-31 |
| **Second regrowth** | 21.0-17.9 | 37.9-43.7 | 4.31-6.28 | 31.5-42.4 | 29-50 |
| **Lucerne** | | | | | |
| **Spring growth** | 25.3-22.4 | 21.4-32.2 | 2.80-4.56 | 17.1-27.3 | 35-51 |
| **Second regrowth** | 20.4-18.6 | 40.2-47.8 | 6.75-8.36 | 30.3-32.5 | 55-67 |

The chemical composition and thus the feeding value of green forages for dairy cows is highly affected by maturity stage at harvest, meaning that farmers can influence the quality substantially by choice of management strategy, however, within a cut, yield and quality are negatively correlated. The variation, which normally occurs in chemical composition and yield within different cuts affected by different harvest dates, is shown for different green forages grown under Danish conditions in Table 2.4. As described in sections 2.2.4 and 2.2.5, stems have a higher cell wall concentration than leaves, because more cells in stems undergo secondary wall thickening and will be lignified with increased maturity. Furthermore, thick lignified cell walls are degraded slower in the rumen and a higher proportion is not digested at all, as described in section 2.4.1. Therefore, within a forage species, the OM digestibility of stem fractions is normally lower than the OM digestibility of leaves fractions, and maturity will affect the digestibility of stem fractions more than the digestibility of leaf fractions (Buxton, 1996). As leaf:stem ratio decreases with advancing maturity, both degradation rate and potential digestibility of NDF decrease as illustrated for perennial ryegrass in Figure 2.10.

### 2.5.1 Grasses versus legumes

Legume leaves are easier to break down than grass leaves when ingested by dairy cows because of more loosely arranged mesophyll cells with more air spaces in legumes leaves compared to grass leaves (section 2.2.4). Furthermore, the angular joints, between veins in

legume leaves due to the netted venation, seem to be weak, and these minor veins can therefore easily fragment into short segments. Opposite, the parallel veins in grass leaves are as long as the length of a leaf blade with no natural breakage points (Wilson and Kennedy, 1996). Therefore, legume leaves require less chewing and rumination activity than grass leaves to fragment into small particles (Kelly and Sinclair, 1989). If the degradation of legume leaves occurs too rapidly it can cause bloat (Wilson, 1994). However, birdsfoot trefoil is less susceptible to leaf and cell rupture than red clover, white clover and lucerne (Lees, 1984).

The lignin concentration, in percentage of both DM and NDF, is higher in legumes than in grasses (ADL, Table 2.4), but as mentioned in section 2.2.4, all lignin in legumes appears in the xylem vascular tissue and no other tissue types contain lignin. The lignin concentration in the xylem of legumes is so high, that the cell walls of these cells are totally indigestible, whereas rumen microorganisms readily and completely can digest the cell walls in the other tissue types (Wilson, 1993; Wilson and Kennedy, 1996; Wilson and Hatfield, 1997). Opposite, the lignin concentration in grasses is lower, but the lignin is scattered between several cell types such as sclerenchyma, parenchyma and xylem. All these cell types are digestible in grasses, but the degradation rate is affected by the lignin (Wilson, 1993; Wilson and Kennedy, 1996). Therefore, the digestion rate of NDF in legumes is higher than in grasses. However, the amount of totally indigestible NDF (iNDF) is higher in legumes than in grasses as the whole xylem vascular tissue constitutes iNDF whereas in grasses, only the thin middle lamella of cells with secondary wall thickening is a truly indigestible fraction besides the lignin (Wilson and Kennedy, 1996; Wilson and Hatfield, 1997). Therefore, potential NDF digestibility in legumes is lower than in grasses, but the degradation rate of potentially digestible NDF is higher as illustrated in Figure 2.10.

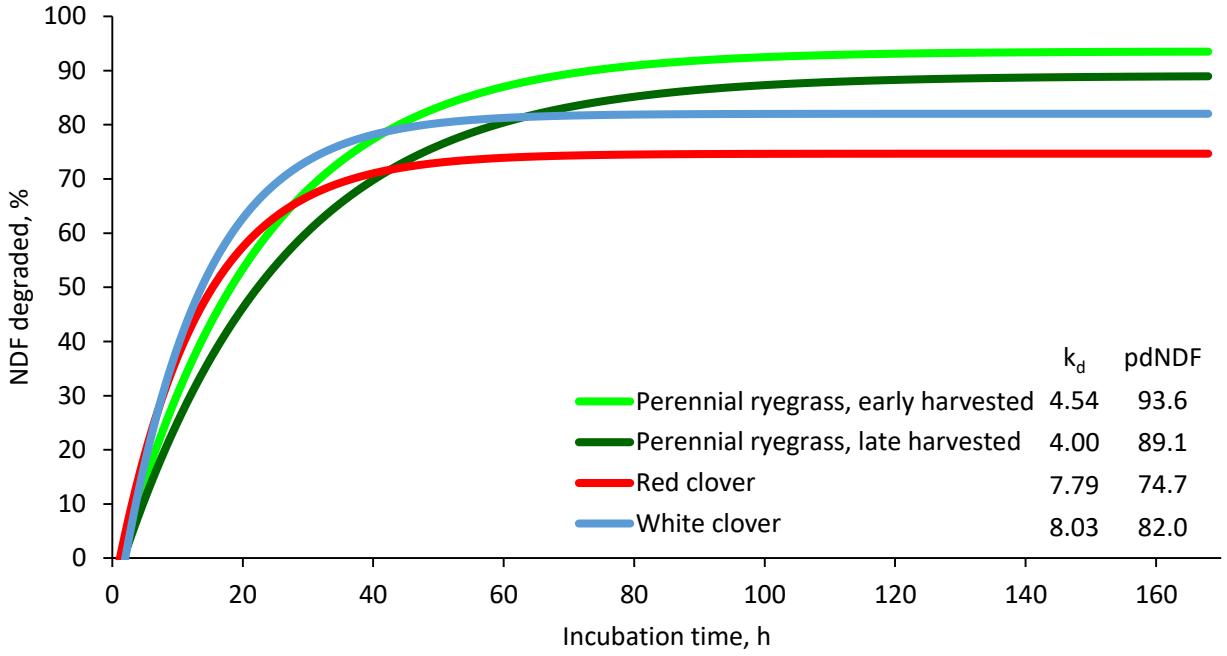


**Figure 2.10.** *In situ* NDF degradation curves for the primary growth of perennial ryegrass harvested at two time points with a 13-days interval, red clover and white clover (Johansen et al., unpublished data). pdNDF is the potentially digestible NDF (% of NDF) and $k_d$ is the digestion rate of pdNDF (%/h).

As legume leaves more easily fragment into small particles than grass leaves, and the cell walls are either totally indigestible (high iNDF:NDF ratio and high specific gravity) or have a high NDF degradation rate, particles of legumes are released faster from the non-escapable rumen pool to the escapable rumen pool than grass particles (section 2.4.1, Figure 2.8). Therefore, the passage rate out of the rumen is higher for legumes than for grasses (Dewhurst *et al.*, 2003b; Kammes and Allen, 2012). Due to the higher NDF degradation rate and the higher passage rate out of the rumen, cows are generally able to eat more legumes than grasses (Steinshamn, 2010).

### 2.5.2 Protein

The CP concentration can vary a lot in green forages depending on harvesting time and N fertilisation, but the CP concentration in grasses and legumes is typically within the range of 100-200 g/kg DM and 180-290 g/kg DM, respectively (Table 2.4) (Weisbjerg *et al.*, 2010). In ensiled green forages, a large part of the true protein is usually converted to soluble NPN due to the activity of plant proteases, however, the magnitude depends on the presence of inhibitory compounds, the pH and the DM concentration (section 2.3). Because of the presence of polyphenol oxidases in red clover, the solubility of CP in red clover silage is normally lower than in other green forage silages. The concentration of soluble NPN can vary from 250-850 g/kg of total N (Rotz and Muck, 1994). As NPN is readily degraded in the rumen (section 2.4.2) the amount of RUP, which contributes to the MP supply, can be minor in green forage silages.

# 3 Objectives and hypotheses

The overall objectives of this PhD thesis were to (i) investigate how different green forages affect feed intake and milk production in dairy cows and (ii) assess how pre-wilting of grass-clover to a higher DM concentration before ensiling affects MP supply to dairy cows. Additionally, the accordance between changes in rumen protein degradation measured *in situ* and *in vivo* was studied and a method to measure field losses during wilting was tested.

The hypotheses of the PhD thesis were:

a. Feed intake and milk production are higher in dairy cows fed legumes than in dairy cows fed grasses when OM digestibility is similar.
b. Milk production in dairy cows fed different grass or legume species reflects forage OM digestibility.
c. Pre-wilting of grass-clover to a higher DM concentration before ensiling will increase the supply of MP to lactating dairy cows.
d. Changes in rumen protein degradation measured *in situ* reflect actual *in vivo* changes.
e. Changes in leaf:stem ratio are a potential tool to estimate field losses when harvesting green forages.

The hypotheses were tested through a meta-analysis and two research experiments (Experiment 1 and 2). Hypotheses (a) and (b) were addressed in the meta-analysis (Paper I) and in Experiment 1 (Paper II), hypotheses (c) and (d) were addressed in Experiment 2 (Paper III-V) and hypothesis (e) was addressed during harvest of forages in Experiment 1 (Paper VI).

Specific objectives:

Paper I: To compare feed intake, milk production, milk composition and OM digestibility in dairy cows fed different grass and legume species in a meta-analysis based on data from the literature.

Paper II: To study how silages of perennial ryegrass, festulolium, tall fescue, red clover and white clover affect feed intake, milk production, digestibility and eating behaviour in dairy cows.

Paper III: To study how pre-wilting of grass-clover to a higher DM concentration before ensiling affects the amount of MP supplied to lactating dairy cows.

Paper IV: To study the AA composition of MP supplied to lactating dairy cows when increasing silage DM concentration.

Paper V: To study whether changes in protein degradation measured *in situ* reflect actual changes *in vivo* in rumen protein degradation when increasing silage DM concentration.

Paper VI: To test the practicability of using changes in leaf:stem ratio in plant material collected in different steps of the harvesting process as a tool to estimate field losses.

# 4 Applied methodology

A meta-analysis and two research experiments were included in the PhD project. The meta-analysis was based on data from 43 published experiments and was used to compare feed intake and milk production in dairy cows fed different grass and legume species. Experiment 1 included 36 intact dairy cows in an 8×8 incomplete Latin square design to study feed intake, milk production and eating behaviour, when feeding different grass and clover species. Experiment 2 included four multi-fistulated cows and was designed to investigate the AA metabolism in the rumen and AA digestion in the small intestine in lactating cows fed grass-clover silages pre-wilted to different DM concentrations.

The current chapter briefly describes and discusses central applied methodologies to justify selected methods. Detailed descriptions of methodologies, experimental procedures, sample collection, chemical analyses and statistical analyses are found in the included papers (Chapter 5), thus references are made to these.

## 4.1 Meta-analysis

Results obtained in a single animal nutrition experiment rarely can be used to make a general conclusion. This is because the hypothesis is often tested on a uniform group of animals in a controlled environment to keep the variability in factors besides those tested, as constant as possible. Furthermore, the feedstuff in question often originates from a single source and does not cover the overall variability of the feedstuff, which especially is a problem when evaluating forages. Therefore, animal nutrition experiments must be repeated by others to verify the observations and to challenge the range of applicability (Sauvant *et al.*, 2008). When several studies on the same subject are published, the obtained knowledge is frequently summarised in a review. However, reviews may be biased as the author has to combine the findings and weight the outcomes from different studies, which is a subjective process (St-Pierre, 2001; Phillips, 2005; Sauvant *et al.*, 2008). Furthermore, when more than 12-15 experiments are involved in a review, it is difficult to differentiate the effect of other factors because of the limitation of the human brain (Sauvant *et al.*, 2008). Instead, statistical methods can be used to summarise and integrate results from individual experiments, and this approach is termed meta-analysis (Glass, 1976). St-Pierre (2001) encourages to use mixed model methodologies in meta-analyses to account for the study effect, and this approach was used in the current meta-analysis (Paper I).

The objective of the current meta-analysis was global hypothesis testing (Sauvant *et al.*, 2008) to test the effect of feeding different green forages to dairy cows on feed intake and milk production. Therefore, the prerequisite for a study to be included in the meta-analysis was that the only difference between diets was the forage source to avoid confounding effects of other diet changes. The standard error of treatment means included in a meta-analysis can differ markedly, as observations derive from different experiments, by which weighting of observations is recommended (St-Pierre, 2001; Sauvant *et al.*, 2008). However, errors given in different publications cannot always be compared, as some report the standard er-

ror of the mean, others report the standard error of the difference or least significant difference and others do not report errors at all. Therefore, observations were weighted by the square rooted number of cows in each treatment mean, giving more weight to observations from larger experiments.

## 4.2 Forage production

The silages used in Experiment 1 (Paper II) were produced from herbages grown on fields at AU Foulum. Perennial ryegrass, festulolium, tall fescue, red clover and white clover were used, as they are the species most relevant for Danish growing conditions. The used variety of festulolium was a cross of annual ryegrass and meadow fescue that was backcrossed to annual ryegrass. The primary growth of all species was harvested and ensiled in spring 2015. The primary growth was used as plant growth is more uniform during spring than during summer, where drought and high temperatures more likely affect the growth. Furthermore, the primary growth accounts for approximately 40% of annual yield per hectare, when four or five cuts are made during a season (Laursen and Petersen, 2010), thus the primary growth is the cut that contributes most to overall feed quality. Perennial ryegrass was mown at two time points with a 13-days interval to obtain variation in maturity within species as well as variation in OM digestibility. The OM digestibility of festulolium and tall fescue was aimed to be within the frame set by the two perennial ryegrass cuts. Half of the perennial ryegrass (early perennial ryegrass), festulolium and tall fescue were mown May 21$^{st}$ and the remaining perennial ryegrass (late perennial ryegrass), red clover and white clover were mown June 3$^{rd}$. Harvest at specific dates ensured some comparability between species, as equal OM digestibility or developmental stage for all species was impossible to reach. After mowing, the herbages were wilted to approximately 350 g DM/kg, before raking, chopping and baling. Further details on field management are described in Paper II. Samples of the forages were taken before mowing, after mowing and after raking, and the leaf:stem ratio was determined in each sample and used to test the practicability of using changes in leaf:stem ratio to estimate field losses (Paper VI). When using changes in leaf:stem ratio to estimate field losses, it is assumed that only leaf material is lost. No real replications of the forages were made in Experiment 1, as each type of forage did not contain variability in terms of source (Udén *et al.*, 2012). Therefore, no general conclusions on single species can be drawn, but only conclusions on the silages actually used. However, studies without real replicates can be used to make general conclusions, when several studies are included in a meta-analysis (Sauvant *et al.*, 2008).

The grass-clover silages used in Experiment 2 (Paper III, IV and V) were produced by two Danish organic farmers, at locations close to Varde and Skjern, respectively, in Western Jutland during the growing season of 2013. Both farmers made a cut of the spring growth and a cut of the first regrowth. For each cut, the DM concentration after wilting was planned to 350 g/kg for half of the herbage and 700 g/kg for the remaining herbage, giving eight silages in total. The approach with two farmers and two cuts within farm was selected to reflect some of the variation in chemical composition that occurs between herbages in prac-

tical farming, due to differences in management, soil types etc. Therefore, in this experiment, more universal conclusions on the effect of DM concentration in grass-clover silage could be made, as four replicates of forage were included.

## 4.3 Animal experiments

A feeding trial with dairy cows can be conducted either as a continuous trial, where a cow is exposed to only one dietary treatment during the entire experiment, or as a change-over trail, where a cow is exposed to at least two dietary treatments during the course of the experiment (Huhtanen and Hetta, 2012). The advantage of a change-over experiment is that the variance between cows is excluded from the residual variance, making the residual error smaller, whereby differences between treatments can be detected with a smaller number of cows. Furthermore, with a given number of cows more treatments can be included in the experiment. According to Huhtanen and Hetta (2012), change-over trials are more precise than, and as accurate as, continuous trials, when differences in animal responses between diets are expected to be small or moderate. However, if the expected difference in DM intake (DMI) between diets exceeds 5 kg/day, change-over experiments can underestimate the difference in production responses (Huhtanen and Hetta, 2012). In feeding trials testing green forage silages, the maximum difference in average DMI between diets is usually below 5 kg/day (e.g. maximum differences in intake: 4.7 kg DM/day (Dewhurst *et al.*, 2003b), 2.7 kg DM/day (Kuoppala *et al.*, 2009), 2.3 kg DM/day (Moorby *et al.*, 2009)). Therefore, change-over designs were applied in both Experiment 1 and 2 to reduce the number of animals and to increase the sensitivity for detecting differences between diets.

### 4.3.1 Adaptation period

When doing feeding and digestibility experiments with dairy cows, it is important to include an adaptation period before making measurements related to the feed in question (Rymer, 2000). The adaptation period ensures steady state in the cow meaning establishment of a steady rumen microflora, a stable daily feed intake and milk production, and to ensure that nutrients excreted in faeces actually originate from the diet currently fed. The time it takes an animal to reach steady state can vary from four to twelve days, depending on the diet and how different the diet is from the previous diet (Rymer, 2000). For change-over experiments with cattle, a 14-day adaptation period is recommended as a minimum (Machado *et al.*, 2016). Therefore, a 17-day adaptation period was used in Experiment 1 (Paper II) and a 14-day adaptation period was used in Experiment 2 (Paper III and IV). Longer adaptation periods will increase experimental costs, but will not increase reliability of measurements. Additionally, longer periods will make the physiological status of individual cows in the first period more different from that in the last period, as lactation stage change during the course of the experiment.

### 4.3.2 Digesta flow and digestibility

Apparent total tract digestibility of feed or of a feed component is defined as the proportion assumed to be absorbed by the animal and is measured as the proportion not excreted

in faeces (Rymer, 2000). When an animal is adapted to a diet and has reached steady state, the direct method to measure apparent total tract digestibility is to record the total amount of feed consumed and to collect all faeces excreted by the animal over a period. The apparent total tract digestibility can then be calculated as

$$\text{Apparent total tract digestibility} = \frac{(\text{Intake (kg/d)} - \text{Faecal output (kg/d)})}{\text{Intake (kg/d)}}$$

Total collection of faeces is time consuming and labour costly, and in lactating dairy cows, the amount and consistency of faeces can be difficult to handle. Furthermore, it can be difficult to separate urine and faeces, which is important to get reliable measurements (Rymer, 2000). As an alternative to the direct method for measurement of digestibility, digestion markers can be used in an indirect method, where only representative spot samples are needed. Markers can occur in the feed naturally (internal marker) or be chemical constituents (external marker) mixed into the feed or pulse dosed to the animal (Ellis *et al.*, 1994). An ideal marker should not be altered or absorbed during the passage through the gastrointestinal tract nor affect the microbial population or digestibility of nutrients. Furthermore, an ideal marker must be associated with the material it is intended to mark, and the method to estimate the marker in collected samples has to be sensitive and specific, and must not interfere with analyses of other nutrients (Faichney, 1993). Even though the ideal marker does not exist (Faichney, 1993), the marker method was used to estimate digesta flow and digestibility in both Experiment 1 (Paper II) and 2 (Paper III and IV). In Experiment 1, apparent total tract digestibility was determined by collection of faecal samples, whereas in Experiment 2, both digesta flow and nutrient digestibility in different segments of the gastrointestinal tract could be determined, as the cows were fitted with cannulas in duodenum and ileum. Assuming that collected samples were representative, digesta flow and faecal output were calculated as (Faichney, 1993):

$$\text{Digesta flow (kg/d)} = \frac{\text{Marker dose rate (g/d)}}{\text{Marker concentration in digesta (g/kg)}}$$

### 4.3.3 Marker allocation

In Experiment 2, chromium oxide ($Cr_2O_3$), titanium oxide ($TiO_2$) and ytterbium chloride ($YbCl_3 \cdot 6H_2O$) were used as external flow markers. $Cr_2O_3$ does not associate with any specific phases of the digesta (Titgemeyer, 1997), $TiO_2$ behaves similarly to $Cr_2O_3$ in the gastrointestinal tract (Myers *et al.*, 2006), whereas $YbCl_3 \cdot 6H_2O$ associates with the particulate phase of the digesta (Teeter *et al.*, 1984). Three markers were used to increase reliability of estimated flows, as the recovery rates are seldom 100% and can vary substantially among animals. For $TiO_2$, Titgemeyer *et al.* (2001) reported an average recovery rate in faeces of 93%, but for individual animals, the recovery rate ranged from 79 to 125%. In other experiments, Titgemeyer *et al.* (2001) reported an average recovery of $TiO_2$ of 95% and 90%, respectively, and an average recovery of $Cr_2O_3$ of 112% and 98%, respectively. Across nine studies, the average recovery of $Cr_2O_3$ was 94% (Titgemeyer, 1997). In the current experiment, markers were dosed into the rumen twice daily, as two daily dosages reduce the diurnal variation in

marker flow compared to a single dosage (Prigge *et al.*, 1981). Especially in duodenal samples, diurnal variation in marker concentration can still occur in spite of a twice-daily dosing regimen (Myers *et al.*, 2006). Therefore, twelve samples were collected from duodenum, ileum and faeces, respectively, at eight-hour intervals for four days, representing every second hour of the day and pooled to get a representative sample accounting for the diurnal variation.

In Experiment 1, $TiO_2$ was used as a digestion marker, as the milk was delivered to the dairy, and we got permission to use $TiO_2$. In this experiment, the marker was mixed into the total mixed ration (TMR), whereby the intake of marker occurred continuously with the intake of TMR. Therefore, a more constant concentration of marker in faeces was expected, than if the marker was dosed only once or twice daily. Faecal samples were collected twice daily, at 8.00h and 14.00h, over three days to get a representative pooled faecal sample.

### 4.3.4 Cannula placement

In digestion experiments with fistulated dairy cows, placement of a cannula in the duodenum is most commonly used to evaluate rumen fermentation, however, cannulas can also be placed in the omasum or abomasum (Harmon and Richards, 1997). Placement of a cannula in the duodenum is favoured compared to placements on the other sites, as the duodenum has a more fixed place in the abdominal cavity and is located close to the body wall which hinders cannula movement, and therefore, fewer postsurgical complications occur (Harmon and Richards, 1997). Therefore, the cows used in Experiment 2 were fistulated in the duodenum. When evaluating forestomach degradation of dietary protein, sampling at the duodenum can cause some errors. Protein in duodenal samples derives from both microbial, feed and endogenous sources. The microbial contribution can be quantified, which in the current experiment was done by separating microbes from the rumen fluid and by using purines as internal markers. Opposite, it is difficult to separate the protein originating from feed and from endogenous sources. The amount of endogenous protein increases as the digesta flows through the stomach, because of sloughed epithelia cells and secretion of gastric juice in the abomasum (Hart and Leibholz, 1990). Furthermore, in the current experiment, where the duodenal cannula was placed approximately 60 cm caudal to the pylorus, endogenous protein from bile will also end up in the duodenal sample (Larsen *et al.*, 2000). Therefore, sampling in the omasum will probably give a more accurate estimate of dietary protein flow out of the rumen than sampling in the duodenum, due to the lower endogenous protein flow (Ahvenjärvi *et al.*, 2000). In the current experiment, the focus was not only on rumen fermentation, but also on MP supply. As endogenous protein contributes to MP supply (section 2.4.2), sampling at duodenum will give more accurate estimates of the digestion of AA in the small intestine, as the protein, including the endogenous protein that enters the small intestine is sampled. However, the obtained estimates will not totally account for the endogenous protein supply, as epithelia cells will also be sloughed in the small intestine. In evaluation of forestomach NDF degradation, duodenal sampling will give more accurate estimates than omasal sampling, as some NDF degradation occurs in the omasum (Ahvenjärvi *et al.*, 2000).

### 4.3.5 Cannula type

In Experiment 2 (Paper III and IV), cows were fitted with simple-T cannulas in the duodenum and ileum. The disadvantage of simple-T cannulas is the difficulty in obtaining representative digesta samples, as not all digesta passing the sampling site will end up in the sample. Collection of total digesta flow is possible with re-entrant cannulas or with closed-T cannulas if properly placed (Harmon and Richards, 1997). However, the advantage of simple-T cannulas to closed-T cannulas is that simple-T cannulas are easier to place, offer fewer postsurgical complications and are less likely to block digesta flow. Furthermore, simple-T cannulas are less disruptive to digesta flow and intestinal motility than closed-T and re-entrant cannulas (Harmon and Richards, 1997). The difficulty in obtaining representative digesta samples with simple-T cannulas is most pronounced if the digesta segregates or tends to be more heterogeneous, which can be a problem particularly in diets with different particle sizes or densities (Harmon and Richards, 1997). In Experiment 2, the cows were fed only grass-clover silages, thus the diet was more homogenous, than if concentrates were also fed, and no dense particles were expected to separate. Therefore, the use of simple-T cannulas was considered appropriate to get representative samples.

### 4.3.6 Feeding practice

Cows were fed ad libitum in both experiments. In Experiment 1, cows were housed in a loose housing system, which can cause competitive eating situations, and thereby affect feed intake of individual cows. This will especially occur, if manger space is limited and all cows are not able to eat at the same time (Albright, 1993). In Experiment 1, all cows were able to eat simultaneously, as one feeding trough (Insentec RIC boxes) was available for each cow, thus the competition was considered as negligible. Each cow had access to one feeding trough only, and therefore did not have the opportunity to select another place to eat, if another cow disturbed during eating. Multiparous cows normally are more dominating than primiparous cows when mixed (Phillips and Rind, 2001). Therefore, primiparous and multiparous cows were grouped separately to reduce dominance hierarchy induced by parity. Within groups, the cows were randomly assigned to a feeding trough not taking dominance relationships between cows into account. When cows can select their eating place freely, they will stand closer to cows of similar rank, whereas the distance to cows with dissimilar ranks will be greater (Manson and Appleby, 1990). Whether the presence of neighbouring cows affected the feeding behaviour of the single cow in the current experiment is unknown, but the cows stayed at the same feeding trough throughout the entire experiment with the same neighbouring cows, thus no confounding effects with treatments occurred. Using the Insentec RIC system allowed measurements of meal size and meal duration besides total feed intake, and these measures were used to evaluate eating behaviour. In Experiment 2, the cows were housed in a tie stall, thus no competition or dominance relationship between cows occurred. The silage was offered in simple mangers, whereby total feed intake was measured only.

# 5 Results

The current section includes the results of the conducted research, which are presented in six papers. A brief summary of the main findings are presented below.

Paper I: *Feed intake and milk production in dairy cows fed different grass and legume species – a meta-analysis*. The meta-analysis based on data from 43 published experiments showed that DMI and milk production are higher in cows fed legume-based diets than in cows fed grass-based diets. Cows fed white clover yielded more milk than cows fed red clover and lucerne, probably because of a higher OM digestibility in white clover. Different grass species similar in OM digestibility resulted in comparable DMI and milk production.

Paper II: *Digestibility and clover proportion determine milk production when silages of different grass and clover species are fed to dairy cows*. Feeding silages of different grass and clover species to dairy cows showed that at comparable silage OM digestibility, inclusion of clover in the diet increased feed intake and ECM production. Differences in ECM yield in cows fed silages of different grass species could be explained by differences in silage OM digestibility. However, cows fed grass silage with a high OM digestibility (83.4%) did not produce the expected amount of ECM based on the amount of OM actually digested in the gastrointestinal tract. Feed intake in cows fed pure white clover was probably regulated physiologically instead of physically, and the eating and drinking behaviour differed markedly from that of cows fed the other silages.

Paper III: *Metabolisable protein supply to lactating dairy cows increased with increasing dry matter concentration in grass-clover silage*. Feeding grass-clover silages with DM concentrations within the range 283-725 g/kg to fistulated, lactating dairy cows showed that increased silage DM concentration increased the amount of AA digested in the small intestine. The increase was caused by a reduced rumen degradation of feed protein, an increased rumen microbial synthesis and an increased small intestinal digestibility of AA. The digestibility of NDF was not affected by silage DM concentration.

Paper IV: *Amino acid profile of metabolisable protein in lactating dairy cows is affected by dry matter concentration in grass-clover silage*. The absolute amount of all individual AA digested in the small intestine increased with increased silage DM concentration when grass-clover silages with different DM concentrations were fed to lactating dairy cows. However, the AA profile of digested AA was negatively affected by increased silage DM concentration, as the proportions of lysine and histidine were reduced.

Paper V: *Comparison of protein degradation in the rumen measured in situ and in vivo*. Changes in effective rumen protein degradation determined using the *in situ* technique caused by increased DM concentrations in grass-clover silages corresponded to *in vivo* changes in rumen protein degradation, when the silages were fed to dairy cows.

Paper VI: *Leaf:stem ratio as a tool to estimate field losses*. The test of the practicability of using changes in leaf:stem ratio as a tool to estimate field losses indicated that the tool can be used to estimate field losses in forages that both have a stem part and leaf part, but the reliability is highly dependent on representative sampling.

## 5.1 Paper I – Feed intake and milk production in dairy cows fed different grass and legume species – a meta-analysis

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# Feed intake and milk production in dairy cows fed different grass and legume species: a meta-analysis

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*The aim of this meta-analysis was to compare feed intake, milk production, milk composition and organic matter (OM) digestibility in dairy cows fed different grass and legume species. Data from the literature was collected and different data sets were made to compare families (grasses* v. *legumes, Data set 1), different legume species and grass family (Data set 2), and different grass and legume species (Data set 3 + 4). The first three data sets included diets where single species or family were fed as the sole forage, whereas the approach in the last data set differed by taking the proportion of single species in the forage part into account allowing diets consisting of both grasses and legumes to be included. The grass species included were perennial ryegrass, annual ryegrass, orchardgrass, timothy, meadow fescue, tall fescue and festulolium, and the legume species included were white clover, red clover, lucerne and birdsfoot trefoil. Overall, dry matter intake (DMI) and milk production were 1.3 and 1.6 kg/day higher, respectively, whereas milk protein and milk fat concentration were 0.5 and 1.4 g/kg lower, respectively, for legume-based diets compared with grass-based diets. When comparing individual legume species with grasses, only red clover resulted in a lower milk protein concentration than grasses. Cows fed white clover and birdsfoot trefoil yielded more milk than cows fed red clover and lucerne, probably caused by a higher OM digestibility of white clover and activity of condensed tannins in birdsfoot trefoil. None of the included grass species differed in DMI, milk production, milk composition or OM digestibility, indicating that different grass species have the same value for milk production, if OM digestibility is comparable. However, the comparison of different grass species relied on few observations, indicating that knowledge regarding feed intake and milk production potential of different grass species is scarce in the literature. In conclusion, different species within family similar in OM digestibility resulted in comparable DMI and milk production, but legumes increased both DMI and milk yield compared with grasses.*

**Keywords:** forage, ruminant, digestibility, feed efficiency, clover

## Implications

Information on expected production responses, when different forages are fed to dairy cows, is important for farmers and advisors in order to optimise forage and milk production. This meta-analysis, based on 43 previous experiments, shows that intake and milk production are higher when cows are fed legume-based diets compared with grass-based diets, and that different grass species similar in digestibility result in comparable intake and milk production. For optimal profitability, harvest yield, digestibility and production costs should be assessed and depending on local conditions, present results show that grass species can be selected freely, and legumes should be included.

## Introduction

In many situations, the main energy source for dairy cows is plant cell walls (Wilson, 1994), but availability of nutrients from cell walls differs depending on their composition and structure (Buxton and Redfearn, 1997). To achieve a high efficiency in the dairy production, it is important to maximise the energy utilisation of the cell wall fraction in the diet (Wilson, 1994). The energy concentration of forages is often reflected in the digestibility, which is a measure of the overall quality of the forage (Allen, 1996). Silages from grasses and legumes constitute usually a large part of the forage in feed rations for dairy cows, but growth of grasses and legumes differs due to seasonal differences, fertilisation strategy and management (Søegaard, 2009; Eriksen *et al.*, 2014). Therefore, knowledge regarding feeding value of different grass and legume species is essential for combined optimisation of forage and milk production. Worldwide, several experiments comparing feed intake and milk production in dairy cows fed different grasses and legumes have been conducted during the last decades, but results from single experiments differ in effect size due to different genetic and physiological status of used animals, different experimental designs, variation in forage quality influenced by cultivation and weather conditions, etc.

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As examples, Vanhatalo *et al*. (2009) reported 1.1 kg/day reduction in total dry matter intake (DMI), whereas Al-Mabruk *et al*. (2004) reported 3.4 kg/day increase in total DMI when cow were fed red clover compared with grass. Therefore, a meta-analysis across experiments will give a more universal answer (Sauvant *et al*., 2008) when evaluating the effect of different grass and legume species on feed intake and milk production in dairy cows.

Steinshamn (2010) has nicely reviewed the effect of forage legumes on feed intake and milk production in dairy cows and concluded that feeding legumes resulted in higher feed intake and milk production compared with grasses. However, variation between experiments was accounted for using *t*-test statistics, and not by using experiment as random in a mixed model procedure as encouraged by St-Pierre (2001). The variance between experiments often exceeds the variance within experiments by which it is important to include the experimental effect in the statistical model (Sauvant *et al*., 2008).

The main objective of this meta-analysis, using mixed modelling procedures, was to compare feed intake and milk production in dairy cows fed different grass and legume species. The hypotheses were that feed intake and milk production are higher for cows fed legumes compared with cows fed grasses and within family, any differences in feed intake and milk production reflect differences in digestibility.

## Material and methods

Published data from experiments with dairy cows fed diets containing grasses and legumes was collected to evaluate how different species affect DMI, milk production and milk composition, and to assess how species differ in organic matter (OM) digestibility. The compilation was done as a mix of database search (CAB Abstracts and Web of Science) and use of reference lists in already collected publications.

Criterion for inclusion of experiments in the meta-analysis was that the forage part of the diets consists solely of grasses, legumes or both. Further, within an experiment, the only difference allowed between diets was the forage source to ensure that responses were caused by the forage source and not by other diet changes. Therefore, within an experiment with total mixed ration (TMR) feeding, the forage : concentrate ratio had to be constant with a similar composition of the concentrate part between diets, and within an experiment using separate allocation of concentrate, all cows had to be offered the same amount of the same concentrate both within and between diets. If other factors were tested within an experiment (e.g. addition of vitamin E or fishmeal, or different levels of concentrate) in addition to the type of forage tested, a random treatment factor was added in the statistical analysis to ensure, that comparisons within an experiment were made between diets only differing in forage source. In addition, all cows should have had *ad libitum* access to the forage or TMR. In a few experiments, the cows were fed restricted in one period and *ad libitum* in another period, then only data from the *ad libitum* fed period was included.

All collected experiments reported data on DMI, milk yield and milk fat and milk protein concentration, whereas only some experiments reported data on milk lactose concentration. For all experiments, energy-corrected milk (ECM, 3.14 MJ/kg) was recalculated using the formula ECM (kg/day) = milk yield (kg/day) × ((38.3 × fat concentration (g/kg) + 24.2 × protein concentration (g/kg) + 16.54 × lactose concentration (g/kg) + 20.7)/3140) if fat, protein and lactose concentrations were given, and the formula ECM (kg/day) = milk yield (kg/day) × ((38.3 × fat concentration (g/kg) + 24.2 × protein concentration (g/kg) + 783.2)/3140) if only fat and protein concentrations were given (Sjaunja *et al*., 1991). For each diet, the feed efficiency was calculated as ECM (kg/day) divided by DMI (kg/day).

Several, but not all experiments reported data regarding OM digestibility of either the forage or the total ration. The method used to determine OM digestibility generally differed between experiments. If various methods were used and reported in the same experiment, the values obtained for the pure forages were used before values obtained for the total ration, and *in vivo* measurements were used before *in vitro* measurements. If *D*-values (digestible OM in DM) were reported, the values were converted to OM digestibility by correcting for the ash concentration.

### *Data sets*

Four data sets were used to maximise statistical power for specific research questions. The purposes with the different data sets were to compare families (Data set 1), to compare different legume species and grass family (Data set 2) and to compare different grass species besides different legume species (Data set 3). The purpose with Data set 4 was the same as Data set 3, but the analytical approach differed allowing experiments with diets including mixes of grasses and legumes to be used to compare species, by which additional experiments could be included in the meta-analysis.

Data set 1 consisted of experiments comparing grasses and legumes in general. Diets with grasses contained either single grass species or mixes of different grass species, and diets with legumes contained either pure white clover (*Trifolium repens* L.), pure red clover (*Trifolium pratense* L.) or pure lucerne (*Medicago sativa* L.). No experiments included diets with mixes of different legume species. The data set included 62 treatment means from 18 experiments in 16 publications. Data on OM digestibility was reported in 15 experiments (52 diets).

Data set 2 consisted of experiments comparing grasses with specific pure legume species or comparing different pure legume species. As in Data set 1, diets with grasses included either single grass species or mixes of different grass species. The diets with specific legume species included pure white clover, pure red clover, pure lucerne or pure birdsfoot trefoil (*Lotus corniculatus* L.). The data set included 90 treatment means from 26 experiments in 21 publications. Data on OM digestibility was reported in 23 experiments (80 diets).

Data set 3 consisted of experiments comparing different pure grass species, comparing specific pure grass species with specific pure legume species or comparing different pure legume species. The specific pure grass species included were perennial ryegrass (*Lolium perenne* L.), orchardgrass (*Dactylis glomerata* L.), timothy (*Phlenum pratense* L.), meadow fescue (*Festuca pratensis* Huds.), tall fescue (*Festuca arundinacea* Schreb.) and festulolium (*Festulolium braunii* K.A.), and the specific pure legume species included white clover, red clover, lucerne and birdsfoot trefoil. The data set included 84 treatment means from 26 experiments in 19 publications. Data on OM digestibility were reported in 20 experiments (65 diets).

Data set 4 consisted of experiments with diets including mixes of grasses and legumes, mixes of different grass species, or pure grass or legume species, taking the proportion of single species in the forage part in each diet into consideration. In most experiments, mixes were made by mixing different species before feeding by which the exact proportions were known. In other experiments, the mixes were grown as mixtures in the field, and proportions based on botanical analysis before harvest were used. For all diets, the legume proportion was known. In 11 out of 43 experiments, botanical information was missing for grass mixtures grown in the field, and proportions based on seeding amount were used if reported (two experiments), otherwise an assumption on equal proportion of grass species was used in the analysis. Some experiments stated the proportion of weed contamination in the forage, and if so, this proportion was included as well. Annual ryegrass (*Lolium multiflorum* Lam.) was included in this data set, besides the species already included in Data set 3, because no available experiments had tested annual ryegrass pure against other pure species, but annual ryegrass was tested pure against mixes and also included in mixes with other species. The data set contained 161 treatment means from 43 experiments in 30 publications.

For all data sets, only diets within an experiment, fulfilling the prerequisites, were used. Therefore, not all diets within an experiment were necessarily included in the data for meta-analysis. If several diets within an experiment included the same forage type, but the forage differed in cut number or variety, these diets were handled as replicates within experiment.

An overview of experiments included in each data set and type of forage included in each experiment is evident from Table 1, and the list of references is given in Supplementary Material S1. The forage : concentrate ratio, DMI and ECM as average across all experiments including additional tested factors were 67 : 33 (43 : 57 to 100 : 0, minimum to maximum), 19.5 kg/day (11.7 to 24.7) and 26.0 kg/day (11.8 to 34.3), respectively. Detailed information on the experiments used in the meta-analysis is given in Supplementary Table S1.

*Statistical analysis*

The statistical analyses were performed using the *lmer* function from the *lme4* package (Bates *et al*., 2015) in R 3.3.1 (R Core Team, 2016).

Data sets 1, 2 and 3 were analysed using the following linear random effect model fitted with restricted maximum likelihood (REML):

$$Y_{ijk} = \mu + \alpha_i + A_j + B_{k(j)} + E_{ijk}$$

where $Y$ is the dependent response variable, $\mu$ the overall mean, $\alpha$ the fixed effect of forage type (Data set 1, $i$ = grasses, legumes; Data set 2, $i$ = grasses, white clover, red clover, lucerne, birdsfoot trefoil; Data set 3, $i$ = perennial ryegrass, orchardgrass, timothy, meadow fescue, tall fescue, festulolium, white clover, red clover, lucerne, birdsfoot trefoil), $A$ the random effect of experiment (Data set 1, $j = 1$ to 18; Data set 2, $j = 1$ to 26; Data set 3, $j = 1$ to 26), $B$ the random effect of additional tested factors within an experiment nested in experiment (for all data sets, $k = 1$ to 3) and $E$ the random residual error assumed to be independent and normal distributed. Residuals were weighted by the square rooted number of cows in each treatment mean. Overall effect of forage type was tested by variance analysis using Satterthwaite approximation for degrees of freedom. Least square means (LSM) and standard error of mean of response variables for the different forage types are presented in Tables 2 to 4. In Data sets 2 and 3, differences between LSM were evaluated using Tukey's method for comparing a family of 5 and 10 estimates, respectively.

Data set 4 was analysed using the following linear regression model with random intercepts fitted with REML:

$$Y_{fjk} = \beta_1 f_1 + \beta_2 f_2 + \beta_3 f_3 + \beta_4 f_4 + \beta_5 f_5 + \beta_6 f_6 + \beta_7 f_7 + \beta_8 f_8 + \beta_9 f_9 + \beta_{10} f_{10} + \beta_{11} f_{11} + \beta_{12} f_{12} + A_j + B_{k(j)} + E_{fjk}$$

where $Y$ is the response variable, $\beta_{1\text{-}12}$ the regression coefficients for the proportion (0 to 1) of perennial ryegrass ($f_1$), annual ryegrass ($f_2$), orchardgrass ($f_3$), timothy ($f_4$), meadow fescue ($f_5$), tall fescue ($f_6$), festulolium ($f_7$), white clover ($f_8$), red clover ($f_9$), lucerne ($f_{10}$), birdsfoot trefoil ($f_{11}$) and weed ($f_{12}$) in the forage part of the diet, respectively, $A$ the random effect of experiment ($j = 1$ to 43), $B$ the random effect of additional tested factors within an experiment nested in experiment ($k = 1$ to 4) and $E$ the random residual error assumed to be independent and normal distributed. Residuals were weighted by the square rooted number of cows in each treatment mean. Weed was included in the analysis with proportions from 0 to 0.3, whereas all other species were included with proportions from 0 to 1. Values presented in Table 5 are the predicted responses, when proportion of the single forage is set to 1 and proportions of all other forages are set to 0, with the standard error of this response in brackets. Differences between values were evaluated by general linear hypothesis testing using the *glht* function from the *multcomp* package (Hothorn *et al*., 2008), and *P*-values were adjusted according to the single step method. Statistical significance was declared by $P \leqslant 0.05$.

**Table 1** *Overview of experiments included in each data set used for meta-analysis and type of forage included in each experiment*

| | Data sets | | | | Type of forage included in experiment | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiments | Data set 1 | Data set 2 | Data set 3 | Data set 4 | Perennial ryegrass | Annual ryegrass | Orchardgrass | Timothy | Meadow fescue | Tall fescue | Festulolium | White clover | Red clover | Lucerne | Birdsfoot trefoil | Weed |
| Al-Mabruk *et al.* (2004) | x | x | x | x | x | | | | | | | | x | | | |
| Andersen *et al.* (2009) | x | x | | x | x | | | | | | x | | | x | | |
| Arvidsson *et al.* (2012) | | | | x | | | | x | | | | | x | | | |
| Baxter *et al.* (1986) (1)[1] | | | x | x | | | x | | | | x | | | | | |
| Baxter *et al.* (1986) (2) | | | x | x | | | x | | | | x | | | | | |
| Bertilsson and Murphy (2003) (1) | x | x | x | x | x | | | | | | | x | x | | | |
| Bertilsson and Murphy (2003) (2) | x | x | x | x | x | | | | | | | x | x | | | |
| Broderick *et al.* (2000) (1) | | x | x | x | | | | | | | | | x | x | | |
| Broderick *et al.* (2000) (2) | | x | x | x | | | | | | | | | x | x | | |
| Broderick *et al.* (2000) (3) | | x | x | x | | | | | | | | | x | x | | |
| Broderick *et al.* (2001) (1) | | | | x | | | | | | | | | x | x | | |
| Broderick *et al.* (2001) (2) | | x | x | x | | | | | | | | | x | x | | |
| Broderick *et al.* (2007) (2) | | x | x | x | | | | | | | | | x | x | | |
| Castle *et al.* (1983) (2) | | | | x | x | | | | | | | x | | | | x |
| Castle *et al.* (1983) (3) | x | x | x | x | x | | | | | | | x | | | | x |
| Dewhurst *et al.* (2003) (1) | x | x | x | x | x | x | | | | | | x | x | x | | |
| Dewhurst *et al.* (2003) (2) | x | x | x | x | x | x | | | | | | x | x | | | |
| Gidlund (2015) | | | | x | | | | x | | | | | x | | | |
| Halmemies-Beauchet-Filleau *et al.* (2014) | x | x | | x | | | | x | x | | | | x | | | |
| Harris *et al.* (1998) (1) | | | | x | x | | | | | | | x | | | | |
| Harris *et al.* (1998) (2) | | | | x | x | | | | | | | x | | | | |
| Heikkilä *et al.* (1992) (1) | | | | x | | | | x | x | | | | x | | | |
| Heikkilä *et al.* (1992) (2) | | | | x | | | | x | x | | | | x | | | |
| Heikkilä *et al.* (1992) (3) | | | | x | | | | x | x | | | | x | | | |
| Heikkilä *et al.* (1996) | | | | x | | x | | x | x | | | | x | | | |
| Hoffman *et al.* (1997) (1) | | x | x | x | | | | | | | | | x | x | | |
| Hoffman *et al.* (1997) (2) | | x | x | x | | | | | | | | | x | x | | |
| Höjer *et al.* (2012) (1) | | | | x | | | | x | x | | | | x | | x | x |
| Höjer *et al.* (2012) (2) | | | | x | x | | | x | x | | | x | x | | | x |
| Hymes-Fecht *et al.* (2013) | | x | x | x | | | | | | | | | x | x | x | |
| Moorby *et al.* (2009) | x | x | x | x | x | | | | | | | | x | | | |
| Orozco-Hernández *et al.* (1997) | x | x | x | x | | | | x | | | | | | x | | |
| Rogers *et al.* (1982) | x | x | x | x | x | | | | | | | x | | | | |
| Rogers *et al.* (1980) | x | x | x | x | x | | | | | | | x | | | | |
| Steinshamn and Thuen (2008) | | | | x | x | | | x | x | | | x | x | | | x |
| Strahan *et al.* (1987) (1) | | | x | x | | | | | | x | x | | | | | |
| Strahan *et al.* (1987) (2) | x | x | x | x | | | | | | x | x | | | x | | |
| Thomas *et al.* (1985) | x | x | x | x | x | | | | | | | | x | | | |
| Tuori and Syrjälä-Qvist (1998) | | | x | x | | | | | x | x | | | | | | |
| Tuori *et al.* (2002) | x | x | x | x | | | | | x | | | | x | | | |
| Vanhatalo *et al.* (2008) | x | x | | x | | | | x | x | | | | x | | | |
| Vanhatalo *et al.* (2009) | x | x | | x | | | | x | x | | | | x | | | |
| Weiss and Shockey (1991) | x | x | x | x | | | x | | | | | | | x | | |

Full list of references is available in Supplementary Material S1 and detailed information on the experiments is given in Supplementary Table S1.
[1]The number in brackets refers to experiment number within publication.

## Results

*Data set 1*

Total DMI and milk yield were higher for cows fed legumes than for cows fed grasses, when comparing grasses with legumes in general (Data set 1, Table 2). The difference in ECM between legumes and grasses (1.0 kg/day) was lower than the difference in milk yield (1.6 kg/day) as both milk fat and milk protein concentrations were lower on legume-based diets than on grass-based diets. No difference was observed in feed efficiency, but OM digestibility was lower for legumes than for grasses.

*Data set 2*

Total DMI of lucerne and red clover were higher than of grasses when comparing grasses and specific legumes (Data set 2, Table 3). Total DMI of white clover and birdsfoot trefoil was not different from the other forages probably due to a smaller number of observations. Numerically, DMI of white clover was comparable with DMI of red clover. Milk yield was highest for white clover and birdsfoot trefoil, in between for red clover and lucerne, and lowest for grasses. No difference was observed in ECM between grasses and red clover, but the remaining three legume species resulted in a higher ECM than grasses, and white clover and birdsfoot trefoil resulted in a higher ECM than red clover. Milk fat concentration was lower for white clover and red clover than for grasses. Red clover resulted in a lower milk protein concentration compared with the other legume species and grasses. Feed efficiency did not differ between forages. The OM digestibility of lucerne was lower than that of red clover, which was lower than the OM digestibility of white clover. The OM digestibility of grasses was numerically in between the OM digestibility of white clover and red clover and higher than that of lucerne. Organic matter digestibility of birdsfoot trefoil did not differ from any of the other forages.

*Data set 3*

No differences in any of the evaluated responses were detected between grass species, when comparing specific grass species and specific legume species (Data set 3, Table 4). Total DMI of red clover, lucerne and birdsfoot trefoil was higher than of perennial ryegrass, whereas DMI of white clover did not differ from the other forages. Milk yield was higher for white clover than for the grass species except timothy and meadow fescue, while milk yield for red clover and lucerne did not differ from the grass species. Milk yield was higher for birdsfoot trefoil than for all grass species. No differences were observed in milk fat concentration between any of the forages. Milk protein concentration was higher for festulolium and lucerne than for red clover. The ECM for white clover was higher than for perennial ryegrass, while

**Table 2** *Effect of forage type (grasses or legumes) on dry matter intake (DMI), milk production and organic matter (OM) digestibility in dairy cows evaluated with Data set 1*

| | Forage type | | |
|---|---|---|---|
| | Grasses | Legumes | *P*-value |
| $n^1$ | 28 | 34 | |
| DMI (kg/day) | 18.3 (0.55) | 19.6 (0.54) | 0.001 |
| Milk yield (kg/day) | 24.5 (1.07) | 26.1 (1.06) | <0.001 |
| Milk fat (g/kg) | 40.3 (0.87) | 38.9 (0.86) | 0.003 |
| Milk protein (g/kg) | 31.6 (0.48) | 31.1 (0.48) | 0.018 |
| ECM[2] (kg/day) | 24.3 (1.21) | 25.3 (1.21) | 0.006 |
| Feed efficiency[3] | 1.33 (0.05) | 1.30 (0.05) | 0.20 |
| $n_{OM}$[4] | 22 | 30 | |
| OM digestibility (%) | 70.4 (0.87) | 67.9 (0.79) | 0.011 |

Least square means given with SEM in brackets.
[1]Number of treatment means included in the analyses of DMI and milk production.
[2]Energy-corrected milk (ECM) (3.14 MJ/kg).
[3]Calculated as kilogram ECM per day divided by kilogram DMI per day.
[4]Number of treatment means included in the analysis of OM digestibility.

**Table 3** *Effect of forage type (grasses, white clover, red clover, lucerne or birdsfoot trefoil) on dry matter intake (DMI), milk production and organic matter (OM) digestibility in dairy cows evaluated with Data set 2*

| | Forage type | | | | | |
|---|---|---|---|---|---|---|
| | Grasses | White clover | Red clover | Lucerne | Birdsfoot trefoil | *P*-value |
| $n^1$ | 28 | 7 | 30 | 22 | 3 | |
| DMI (kg/day) | 18.9 (0.48)$^{b}$ | 20.0 (0.71)$^{ab}$ | 20.0 (0.47)$^{a}$ | 21.0 (0.50)$^{a}$ | 21.8 (1.21)$^{ab}$ | <0.001 |
| Milk yield (kg/day) | 26.2 (0.97)$^{c}$ | 29.6 (1.07)$^{a}$ | 27.3 (0.97)$^{b}$ | 27.7 (0.98)$^{b}$ | 31.4 (1.39)$^{a}$ | <0.001 |
| Milk fat (g/kg) | 39.8 (0.73)$^{a}$ | 37.2 (0.96)$^{b}$ | 38.1 (0.71)$^{b}$ | 39.1 (0.74)$^{ab}$ | 38.7 (1.56)$^{ab}$ | 0.001 |
| Milk protein (g/kg) | 31.6 (0.38)$^{a}$ | 31.8 (0.46)$^{a}$ | 30.8 (0.38)$^{b}$ | 31.3 (0.39)$^{a}$ | 31.3 (0.67)$^{ab}$ | <0.001 |
| ECM[2] (kg/day) | 25.7 (0.99)$^{d}$ | 28.1 (1.12)$^{ab}$ | 26.1 (0.99)$^{cd}$ | 27.0 (1.00)$^{bc}$ | 30.4 (1.51)$^{a}$ | <0.001 |
| Feed efficiency[3] | 1.35 (0.04) | 1.39 (0.05) | 1.31 (0.04) | 1.30 (0.04) | 1.43 (0.08) | 0.07 |
| $n_{OM}$[4] | 22 | 6 | 28 | 21 | 3 | |
| OM digestibility (%) | 71.5 (1.17)$^{ab}$ | 73.6 (1.64)$^{a}$ | 69.4 (1.12)$^{b}$ | 66.0 (1.17)$^{c}$ | 67.7 (2.62)$^{abc}$ | <0.001 |

Least square means given with SEM in brackets.
[a,b,c,d]Values in same line with different letters differ, $P < 0.05$.
[1]Number of treatment means included in the analyses of feed intake and milk production.
[2]Energy-corrected milk (ECM) (3.14 MJ/kg).
[3]Calculated as kilogram ECM per day divided by kilogram DMI per day.
[4]Number of treatment means included in the analysis of OM digestibility.

**Table 4** *Effect of forage type (perennial ryegrass, orchardgrass, timothy, meadow fescue, tall fescue, festulolium, white clover, red clover, lucerne or birdsfoot trefoil) on dry matter intake (DMI), milk production and organic matter (OM) digestibility in dairy cows evaluated with Data set 3*

| | Forage type | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Perennial ryegrass | Orchardgrass | Timothy | Meadow fescue | Tall fescue | Festulolium | White clover | Red clover | Lucerne | Birdsfoot trefoil | *P*-value |
| $n$[1] | 9 | 5 | 3 | 2 | 3 | 8 | 7 | 23 | 21 | 3 | |
| DMI (kg/day) | 17.6 (0.62)$^{b}$ | 18.5 (0.84)$^{ab}$ | 19.6 (0.92)$^{ab}$ | 18.8 (1.16)$^{ab}$ | 17.6 (1.02)$^{ab}$ | 18.6 (0.83)$^{ab}$ | 19.4 (0.66)$^{ab}$ | 19.8 (0.49)$^{a}$ | 20.8 (0.50)$^{a}$ | 21.3 (1.04)$^{a}$ | <0.001 |
| Milk yield (kg/day) | 25.2 (1.09)$^{c}$ | 25.1 (1.23)$^{c}$ | 26.8 (1.27)$^{bc}$ | 24.8 (1.47)$^{bc}$ | 24.1 (1.37)$^{c}$ | 24.6 (1.26)$^{c}$ | 28.4 (1.12)$^{ab}$ | 26.3 (1.03)$^{c}$ | 26.9 (1.03)$^{bc}$ | 30.6 (1.37)$^{a}$ | <0.001 |
| Milk fat (g/kg) | 39.7 (1.07) | 41.1 (1.39) | 39.9 (1.48) | 41.1 (1.86) | 41.3 (1.66) | 41.9 (1.39) | 37.1 (1.13) | 38.1 (0.89) | 39.3 (0.91) | 39.0 (1.68) | 0.025[5] |
| Milk protein (g/kg) | 31.2 (0.48)$^{ab}$ | 32.2 (0.60)$^{ab}$ | 31.5 (0.63)$^{ab}$ | 31.8 (0.77)$^{ab}$ | 32.3 (0.70)$^{ab}$ | 32.6 (0.61)$^{a}$ | 31.6 (0.51)$^{ab}$ | 30.7 (0.43)$^{b}$ | 31.4 (0.43)$^{a}$ | 31.3 (0.70)$^{ab}$ | 0.011 |
| ECM[2] (kg/day) | 24.6 (1.11)$^{c}$ | 25.0 (1.30)$^{bc}$ | 26.1 (1.35)$^{abc}$ | 24.9 (1.61)$^{abc}$ | 24.1 (1.48)$^{bc}$ | 24.7 (1.32)$^{bc}$ | 26.9 (1.14)$^{ab}$ | 25.0 (1.02)$^{bc}$ | 26.2 (1.03)$^{abc}$ | 29.7 (1.48)$^{a}$ | 0.001 |
| Feed efficiency[3] | 1.38 (0.05)$^{a}$ | 1.31 (0.06)$^{ab}$ | 1.34 (0.06)$^{ab}$ | 1.31 (0.08)$^{ab}$ | 1.32 (0.07)$^{ab}$ | 1.27 (0.06)$^{ab}$ | 1.39 (0.05)$^{a}$ | 1.28 (0.04)$^{b}$ | 1.28 (0.04)$^{ab}$ | 1.41 (0.07)$^{ab}$ | 0.014 |
| $n_{OM}$[4] | 6 | 3 | 3 | 2 | 1 | 0 | 6 | 21 | 20 | 3 | |
| OM digestibility (%) | 71.4 (1.71)$^{a}$ | 69.4 (2.73)$^{ab}$ | 68.6 (2.34)$^{ab}$ | 71.3 (3.20)$^{ab}$ | 70.1 (4.59)$^{ab}$ | — | 73.6 (1.74)$^{a}$ | 69.2 (1.29)$^{a}$ | 65.6 (1.35)$^{b}$ | 67.1 (2.63)$^{ab}$ | <0.001 |

Least square means given with SEM in brackets.
[a,b,c]Values in same line with different letters differ, $P < 0.05$.
[1]Number of treatment means included in the analyses of feed intake and milk production.
[2]Energy-corrected milk (ECM) (3.14 MJ/kg).
[3]Calculated as kilogram ECM per day divided by kilogram DMI per day.
[4]Number of treatment means included in the analysis of OM digestibility.
[5]The overall statistical test gave a significant result, whereas the Tukey method for comparing differences between means did not gave any significant differences.

**Table 5** *Predicted effect of forage type (perennial ryegrass, annual ryegrass, orchardgrass, timothy, meadow fescue, tall fescue, festulolium, white clover, red clover, lucerne or birdsfoot trefoil), when proportion of the single forage is set to 1 and proportions of all other forages are set to 0, on dry matter intake (DMI) and milk production in dairy cows evaluated with Data set 4*

| | Forage type | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Perennial ryegrass | Annual ryegrass | Orchardgrass | Timothy | Meadow fescue | Tall fescue | Festulolium | White clover | Red clover | Lucerne | Birdsfoot trefoil | Weed |
| $n$[1] | 41 | 8 | 5 | 40 | 28 | 4 | 9 | 26 | 63 | 23 | 4 | 15 |
| DMI (kg/day) | 17.7 (0.56)$^{c}$ | 18.1 (1.05)$^{abc}$ | 18.7 (0.86)$^{abc}$ | 19.9 (0.68)$^{abc}$ | 18.9 (0.83)$^{abc}$ | 17.8 (1.02)$^{abc}$ | 18.9 (0.84)$^{abc}$ | 19.2 (0.62)$^{abc}$ | 19.5 (0.43)$^{b}$ | 20.6 (0.46)$^{a}$ | 21.2 (1.05)$^{abc}$ | 19.4 (4.12)$^{abc}$ |
| Milk yield (kg/day) | 25.4 (0.97)$^{cd}$ | 24.4 (1.24)$^{cd}$ | 25.2 (1.14)$^{cd}$ | 26.2 (1.03)$^{bcd}$ | 23.3 (1.12)$^{d}$ | 23.9 (1.25)$^{cd}$ | 25.0 (1.15)$^{cd}$ | 28.5 (0.99)$^{ab}$ | 26.1 (0.90)$^{c}$ | 26.7 (0.92)$^{c}$ | 30.3 (1.26)$^{a}$ | 19.6 (3.89)$^{abcd}$ |
| Milk fat (g/kg) | 40.4 (0.99)$^{ab}$ | 40.4 (1.51)$^{ab}$ | 42.5 (1.32)$^{ab}$ | 41.2 (1.11)$^{ab}$ | 42.8 (1.27)$^{a}$ | 42.8 (1.51)$^{ab}$ | 43.1 (1.32)$^{a}$ | 38.7 (1.05)$^{b}$ | 39.9 (0.87)$^{ab}$ | 41.0 (0.90)$^{ab}$ | 40.6 (1.53)$^{ab}$ | 55.0 (5.47)$^{ab}$ |
| Milk protein (g/kg) | 31.9 (0.41)$^{ab}$ | 32.8 (0.58)$^{ab}$ | 32.8 (0.52)$^{a}$ | 32.4 (0.45)$^{a}$ | 32.6 (0.50)$^{ab}$ | 32.9 (0.58)$^{ab}$ | 33.2 (0.52)$^{a}$ | 32.2 (0.42)$^{ab}$ | 31.5 (0.36)$^{b}$ | 32.1 (0.37)$^{a}$ | 32.0 (0.59)$^{ab}$ | 33.8 (2.03)$^{ab}$ |
| ECM[2] (kg/day) | 25.1 (0.93)$^{cd}$ | 24.3 (1.29)$^{bc}$ | 25.7 (1.15)$^{bc}$ | 26.1 (1.01)$^{bc}$ | 24.0 (1.12)$^{cd}$ | 24.5 (1.29)$^{bc}$ | 25.7 (1.16)$^{bc}$ | 27.4 (0.96)$^{ab}$ | 25.5 (0.84)$^{c}$ | 26.6 (0.86)$^{bd}$ | 29.9 (1.30)$^{a}$ | 23.5 (4.37)$^{abcd}$ |
| Feed efficiency[3] | 1.36 (0.04) | 1.39 (0.07) | 1.33 (0.06) | 1.34 (0.05) | 1.31 (0.05) | 1.34 (0.07) | 1.31 (0.06) | 1.40 (0.04) | 1.33 (0.03) | 1.30 (0.03) | 1.42 (0.07) | 1.09 (0.27) |

Standard errors given in brackets.
[a,b,c,d]Values in same line with different letters differ, $P < 0.05$.
[1]Number of treatments in which the forage type is included with a proportion above 0. Total $n = 161$. Weed was included in the analysis with proportions from 0 to 0.3, whereas all other species were included with proportions from 0 to 1.
[2]Energy-corrected milk (ECM) (3.14 MJ/kg).
[3]Calculated as kilogram ECM per day divided by kilogram DMI per day.

ECM for birdsfoot trefoil was higher than for red clover and the grass species except timothy and meadow fescue. No other differences in ECM were detected between the forage species. Perennial ryegrass and white clover resulted in a higher feed efficiency than red clover. The OM digestibility of perennial ryegrass, white clover and red clover was higher than of lucerne, with no difference in OM digestibility between the other forages.

*Data set 4*

When comparing different forages using the data set taking the proportion of each single species into account, no differences were observed between any of the included grass species (Data set 4, Table 5). Further, weed did not differ from any of the cultivated forage species in any of the evaluated responses, probably due to a high variation in the estimates for weed derived from proportions in the model only varying from 0 to 0.3. Nevertheless, milk yield was numerically lower (3.7 to 10.7 kg/day) for weed than for the other forage species. Total DMI of lucerne was higher than of red clover, which was higher than DMI of perennial ryegrass. Meadow fescue resulted in a lower milk yield than red clover and lucerne, which both resulted in a lower milk yield than white clover and birdsfoot trefoil. Milk yield was higher for birdsfoot trefoil and white clover than for all grass species, except timothy for white clover. Milk fat concentration was lower for white clover than for meadow fescue and festulolium, and milk protein concentration was lower for red clover than for orchardgrass, timothy, festulolium and lucerne. Perennial ryegrass and meadow fescue resulted in lower ECM than white clover and birdsfoot trefoil, and red clover resulted in lower ECM than the other included legume species. The ECM was higher for birdsfoot trefoil than for lucerne. No differences in feed efficiency were detected between any of the forages.

## Discussion

*Grasses* v. *legumes*

Legume-based diets resulted in higher DMI and milk yield than grass-based diets when evaluated with Data set 1, but the difference between legumes and grasses was also evident when using the other data sets. Legumes contain less fibre than grasses but the fibre in legumes is generally more lignified (Buxton and Redfearn, 1997). Lignin is resistant to rumen digestion and is the main factor, which affects digestibility of cellulose, as lignin acts as a physical barrier limiting the microbes' access to cellulose (Van Soest *et al.*, 1978). In legumes, it is only xylem and tracheary cells, which are lignified, whereas lignin also occurs in several other cell types, such as sclerenchyma and parenchyma, in grasses. Cells, which are lignified in legumes, are indigestible, whereas lignified cells in grasses are digestible to some extent making rumen digestion rate of potential digestible fibre higher for legumes than for grasses (Buxton and Redfearn, 1997). Further, the rumen passage rate is higher for legumes than for grasses (Dewhurst *et al.*, 2003). The difference in fibre composition and passage rate can explain the higher DMI on legume-based diets compared with grass-based diets, despite the lower OM digestibility for legumes than for grasses. The higher DMI for legumes was reflected in the higher milk production, as no difference in feed efficiency between grass- and legume-based diets was detected.

*Legume species*

In Data sets 2, 3 and 4, the DMI of red clover and white clover was comparable, but white clover resulted in a higher milk yield and ECM, probably because of a higher OM digestibility in white clover compared with red clover. Higher digestibility enhances the energy intake from the forage, which causes a higher milk yield at comparable feed intake levels. This was also expressed in a higher feed efficiency for white clover than for red clover in Data set 3, even though the difference in feed efficiency only was numerical in Data sets 2 and 4. The difference in OM digestibility between white clover and red clover can be caused by differences in morphological growth. White clover has a stoloniferous growth, meaning that stem and stolon are growing along the soil surface (Black *et al.*, 2009), and no stems will end up in the material used for feeding as long as white clover is in the vegetative stage. However, flowering in white clover will increase the lignin concentration substantially with a reduced OM digestibility in consequence (Weisbjerg *et al.*, 2010). In contrast, red clover has a vertical positioned growth with stems growing upwards, by which stems will be harvested when cutting. The concentration of NDF is twice as high in the stems of legumes as in the leaf blade (Buxton and Redfearn, 1997).

The DMI of lucerne was higher than that of red clover when evaluated in Data set 4, but the same numerical difference between lucerne and red clover appeared as a tendency ($P < 0.1$, data not shown) in Data sets 2 and 3. In Data set 4, lucerne also resulted in a higher ECM compared with red clover, with the same numerical difference in Data sets 2 and 3. None of the data sets showed a difference in feed efficiency between red clover and lucerne, indicating that the higher milk yield is due to the higher DMI. However, the OM digestibility was lower for lucerne than for red clover (Data sets 2 and 3), by which the energy intake between the two diets was comparable, and therefore a difference in milk yield was not expected. In this meta-analysis, BW changes were not considered, but some studies showed a lower BW gain in cows fed lucerne compared with cows fed red clover (Broderick *et al.*, 2000 and 2001 and 2007), resulting in more energy available for milk production.

Birdsfoot trefoil was not different from the other legume species in DMI, but was superior to red clover and lucerne in milk yield and ECM in both Data sets 2, 3, and 4. Woodward *et al.* (2000) showed that the increased milk yield for birdsfoot trefoil is due to the activity of condensed tannins, and the effect is proportional to the concentration (Hymes-Fecht *et al.*, 2013).

Overall, both milk fat and milk protein concentrations were lower on legume-based diets compared with grass-based diets (Data set 1). However, when evaluating specific

legume species in Data set 2, none of the legumes species differed in milk fat concentration, but only white clover and red clover were lower than grasses. As Steinshamn (2010) reviewed, the lower milk fat concentration on legume-based diets is probably caused by an inhibition of the milk fat synthesis due to the combined effect of some intermediates from the bio-hydrogenation pathway and an increased supply of long-chain fatty acids to the mammary gland when cows are fed legumes compared with grasses. For milk protein concentration, red clover was lower than grasses, white clover and lucerne, whereas white clover and lucerne did not differ from grasses (Data set 2). The reduced milk protein concentration for red clover may be related to the presence of polyphenol oxidases in red clover, which can form complexes with plant proteins and protect proteins from degradation in the rumen (Lee, 2014). However, these polyphenol oxidases can also affect bioavailability of sulphur containing amino acids (Lee, 2014), resulting in a reduced apparent total tract digestibility and a reduced plasma concentration of methionine in cows fed a red clover diet compared with a grass diet (Lee *et al*., 2009; Vanhatalo *et al*., 2009), and methionine can be limiting for milk protein synthesis.

*Grass species*

No differences were observed between grass species in any of the evaluated response parameters, neither when evaluated using Data set 3 nor Data set 4. For many of the grass species, there were only few observations where the species were fed pure (Data set 3), and this reduced the strength of the estimates. Further, only five of the included experiments from three publications have compared different pure grass species within experiment, which shows that knowledge regarding feed intake and milk production potential of different grass species is scarce in the literature.

Developmental stage of grass species at harvest will most probably affect the feeding value, as harvest date, and consequently developmental stage, has a substantial effect on chemical composition and digestibility (Weisbjerg *et al*., 2010). Digestibility is more comparable between experiments than developmental stage. As OM digestibility did not differ between the evaluated grass species, differences in DMI and milk production were not expected either. This indicated, for this level of OM digestibility, that different grass species have the same value for milk production.

Half of the experiments, where grass species were fed as the sole forage, were conducted before 1990. As breeding continuously improve quality traits regarding feeding and cultivation, the varieties included in the current meta-analysis are probably not representative for those used today. In north-western Europe, DM yield for perennial ryegrass has increased 4% to 5% per decade, whereas the improvement in digestibility is uncertain (Wilkins and Humphreys, 2003; McDonagh *et al*., 2016). However, increased OM digestibility will affect level of DMI and milk yield positively, but the effect at an increased OM digestibility will most likely not differ between grass species.

*Different approaches to the analysis*

When using the approach taking the proportion of single species in mixes into account, as done in Data set 4, the number of treatment means used to predict the responses increased; especially for perennial ryegrass, timothy, meadow fescue, white clover and red clover, whereas the numbers only increased slightly for the other included species. Further, Data set 4 resulted in values for annual ryegrass and weed, as these only were fed in mixes with other species. For almost all parameters, the standard error of the estimates decreased when using Data set 4 compared with using Data set 3, indicating that linear regression including mixes strengthen the estimates. The reduced variation indicated that the variance around a linear relationship between the evaluated response parameters and the proportion of single species was low.

The DMI was on average predicted 0.2 kg/day higher for the grass species and 0.2 kg/day lower for the legume species, when using Data set 4 compared with using Data set 3. Contrary, ECM was on average across all species predicted 0.3 kg/day higher in Data set 4 than in Data set 3. The high level of agreement between the estimates using the two different approaches indicated that the response in DMI is linearly correlated to the proportion of single species. The increase in ECM from Data set 3 to Data set 4 could be caused by positive interactions between species. Moorby *et al*. (2009) and Halmemies-Beauchet-Filleau *et al*. (2014) both reported a higher ECM for cows fed mixtures containing 33% or 67% red clover, compared with cows fed pure grass or pure red clover. The difference in estimated DMI between Data sets 3 and 4 cannot be explained by same positive interaction as for ECM, as this should have increased DMI for both grasses and legumes in Data set 4 compared with Data set 3, and not decreased DMI for the legume species. According to Huhtanen *et al*. (2007), silage intake of dairy cows can only be predicted with reasonable accuracy if legume proportion is $<0.5$, presumably because of changed mechanism for regulation of feed intake for legume silages compared with grass silages. Whether this can explain the observed difference in DMI predictions between Data sets 3 and 4 is unknown.

For all included species, both milk fat and milk protein concentration were predicted higher (on average 1.5 and 0.7 g/kg, respectively) when using Data set 4 compared with using Data set 3. Whether these increases were due to more balanced diets, for example, fatty acid or amino acid profiles, when feeding more than one species at a time, or were caused by higher milk fat and milk protein concentrations due to genetic status of cows in the additional experiments included in Data set 4 compared with the experiments already included in Data set 3, is unknown. However, individual experiments do not indicate that mixed diets should be superior to diets of pure species regarding milk protein and milk fat concentration (Bertilsson and Murphy, 2003; Vanhatalo *et al*., 2009).

*Organic matter digestibility*

One of the intentions with this meta-analysis was to relate DMI and ECM to OM digestibility to determine the impact of

increasing OM digestibility, and to compare the individual species at equal OM digestibility. However, a regression of DMI or ECM on OM digestibility was not possible with the data available, due to a lack of variation in parameters within grasses and legumes within experiment. A random regression within experiments across grasses and legumes would result in incorrect estimates as legumes generally resulted in higher DMI and ECM than grasses. On the contrary, a regression of feed efficiency on OM digestibility was possible across grasses and legumes, as Data set 1 showed that feed efficiency did not differ between grasses and legumes. The regression was conducted including a random slope within experiment. For Data sets 1, 2, 3 and 4 the feed efficiency increased by 0.009 kg ECM/kg DMI ($P = 0.08$), 0.006 kg ECM/kg DMI ($P = 0.02$), 0.007 kg ECM/kg DMI ($P = 0.007$) and 0.004 kg ECM/kg DMI ($P = 0.14$), with each percentage point increase in OM digestibility. Converted, the responses corresponded to 0.1 to 0.2 kg ECM/day with each percentage point increase in OM digestibility, which illustrated the importance of a high OM digestibility.

*Conclusion*

This meta-analysis confirmed that DMI and milk production is higher for cows fed legume-based diets compared with cows fed grass-based diets, and the milk yield reflected the intake of DM. Cows fed legumes yielded milk with a lower fat concentration compared with cows fed grasses, whereas the milk protein concentration only was lowered in cows fed red clover. White clover resulted in higher milk yield than red clover and alfalfa, probably due to higher OM digestibility. Different grass species similar in OM digestibility resulted in comparable DMI and milk production.

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## Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731117001215

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## Feed intake and milk production in dairy cows fed different grass and legume species – a meta-analysis

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## Supplementary Material S1

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## Supplementary Table S1

***Table S1*** Description of experiments used in the meta-analysis

| Experiment | Location | Feeding | Breed | DIM[2] | Additional tested factors | Forage %[3] | DMI[4] | MY[5] | ECM[6] |
|---|---|---|---|---|---|---|---|---|---|
| Al-Mabruk et al. (2004) | Wales | Separate | Holstein-Friesian | 77 | - E vitamin | 62 | 17.7 | 24.0 | 22.2 |
| | | | | | + E vitamin | 61 | 18.1 | 24.4 | 22.3 |
| Andersen et al. (2009) | Denmark | TMR | Danish Holstein | EL[7] | | 59 | 22.0 | 33.8 | 34.3 |
| Arvidsson et al. (2012) | Sweden | Separate | Swedish Red | 196 | | 63 | 16.1 | 20.9 | 23.6 |
| Baxter et al. (1986) (1)[1] | Kentucky, US | Separate | Jersey | EL | | 64 | 17.3 | 17.9 | 19.8 |
| Baxter et al. (1986) (2) | Kentucky, US | Separate | Jersey | EL | | 73 | 14.8 | 18.8 | 21.2 |
| Bertilsson and Murphy (2003) (1) | Sweden | Separate | Swedish Red and White | 50 | | 66 | 21.0 | 28.7 | 28.7 |
| Bertilsson and Murphy (2003) (2) | Sweden | Separate | Swedish Red and White | 82 | | 68 | 22.1 | 29.5 | 30.6 |
| Broderick et al. (2000) (1) | Wisconsin, US | TMR | Holstein | 57 | - fishmeal | 71 | 21.0 | 33.6 | 30.5 |
| | | | | | + fishmeal | 71 | 22.0 | 34.3 | 30.8 |
| Broderick et al. (2000) (2) | Wisconsin, US | TMR | Holstein | 42 | - fishmeal | 64 | 19.7 | 29.7 | 26.9 |
| | | | | | + fishmeal | 64 | 20.3 | 31.9 | 28.8 |
| Broderick et al. (2000) (3) | Wisconsin, US | TMR | Holstein | 59 | - fishmeal | 60 | 22.9 | 32.6 | 29.8 |
| | | | | | + fishmeal | 60 | 22.8 | 34.3 | 31.1 |
| Broderick et al. (2001) (1) | Wisconsin, US | TMR | Holstein | 65 | | 60 | 23.6 | 33.2 | 30.5 |
| Broderick et al. (2001) (2) | Wisconsin, US | TMR | Holstein | 146 | | 61 | 22.7 | 30.4 | 31.0 |
| Broderick et al. (2007) (2) | Wisconsin, US | TMR | Holstein | 192 | | 51 | 23.5 | 29.6 | 29.0 |
| Castle et al. (1983) (2) | Scotland | Separate | Ayrshire | 42 | | 68 | 15.7 | 21.1 | 20.4 |
| Castle et al. (1983) (3) | Scotland | Separate | Ayrshire | 28 | | 64 | 14.5 | 20.2 | 20.1 |
| Dewhurst et al. (2003) (1) | Wales | Separate | Holstein-Friesian | 64 | | 65 | 19.8 | 28.1 | 29.6 |
| Dewhurst et al. (2003) (2) | Wales | Separate | Holstein-Friesian | 82 | 4 kg con[8] | 81 | 18.4 | 24.3 | 22.8 |
| | | | | | 8 kg con | 68 | 21.1 | 29.9 | 28.3 |
| Gidlund (2015) | Sweden | TMR | Swedish Red and White | NR[9] | 15.2% CP in TMR | 60 | 19.7 | 27.2 | 28.1 |
| | | | | | 16.8% CP in TMR | 60 | 21.4 | 29.4 | 30.2 |
| | | | | | 18.3% CP in TMR | 60 | 23.1 | 30.3 | 30.7 |
| | | | | | 20.0% CP in TMR | 60 | 23.6 | 29.6 | 30.0 |
| Halmemies-Beauchet-Filleau et al. (2014) | Finland | TMR | Finnish Ayrshire | 108 | | 60 | 19.6 | 28.0 | 28.1 |
| Harris et al. (1998) (1) | New Zealand | Separate | Jersey | 115 | | 100 | 12.0 | 12.0 | 15.0 |
| Harris et al. (1998) (2) | New Zealand | Separate | Jersey and Friesian | 246 | | 100 | 11.7 | 9.4 | 11.8 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Heikkilä et al. (1992) (1) | Finland | Separate | Finnish Ayrshire | NR | | 65 | 21.5 | 28.2 | 30.6 |
| Heikkilä et al. (1992) (2) | Finland | Separate | Finnish Ayrshire | NR | | 61 | 19.3 | 25.3 | 26.2 |
| Heikkilä et al. (1992) (3) | Finland | Separate | Finnish Ayrshire | NR | | 55 | 20.7 | 31.2 | 31.0 |
| Heikkilä et al. (1996) | Finland | Separate | Finnish Ayrshire | NR | | 57 | 19.8 | 29.0 | 29.5 |
| Hoffman et al. (1997) (1) | Wisconsin, US | TMR | Holstein | 60 | Early cut, 16% CP in con | 58 | 21.8 | 33.6 | 31.7 |
| | | | | | Late cut, 22.5% CP in con | 58 | 20.2 | 30.1 | 28.5 |
| Hoffman et al. (1997) (2) | Wisconsin, US | TMR | Holstein | 70 | | 50 | 19.7 | 30.5 | 29.9 |
| Höjer et al. (2012) (1) | Sweden | Separate | Swedish Red | 130 | | 74 | 20.1 | 25.9 | 27.6 |
| Höjer et al. (2012) (2) | Norway | Separate | Norwegian Red | 129 | | 76 | 23.0 | 26.3 | 27.5 |
| Hymes-Fecht et al. (2013) | Wisconsin, US | TMR | Holstein | 161 | | 60 | 24.7 | 33.6 | 32.0 |
| Moorby et al. (2009) | Wales | Separate | Holstein-Friesian | 103 | | 81 | 18.0 | 26.0 | 24.1 |
| Orozco-Hernández et al. (1997) | Canada | Separate | Holstein | 104 | No barley | 99 | 19.3 | 23.9 | 24.6 |
| | | | | | + 17% barley | 82 | 20.3 | 23.6 | 25.3 |
| | | | | | + 34% barley | 65 | 19.9 | 22.6 | 24.6 |
| Rogers et al. (1982) | Australia | Separate | Friesian | EL | | 100 | 14.0 | 14.6 | 14.0 |
| Rogers et al. (1980) | Australia | Separate | NR | NR | | 100 | 17.9 | 18.6 | 17.3 |
| Steinshamn and Thuen (2008) | Norway | Separate | Norwegian Red | 74 | No con | 98 | 14.4 | 22.1 | 20.5 |
| | | | | | 10 kg con | 60 | 19.8 | 28.0 | 27.5 |
| Strahan et al. (1987) (1) | Kentucky, US | Separate | Holstein | 60 | | 56 | 16.9 | 20.5 | 18.7 |
| Strahan et al. (1987) (2) | Kentucky, US | Separate | Holstein | 60 | | 59 | 18.9 | 19.5 | 17.7 |
| Thomas et al. (1985) | England | Separate | British Friesian | EL | | 58 | 16.6 | 25.7 | 24.5 |
| Tuori and Syrjälä-Qvist (1998) | Finland | Separate | Finnish Ayrshire | NR | | 53 | 17.2 | 27.2 | 28.7 |
| Tuori et al. (2002) | Finland | Separate | Ayrshire | NR | | 56 | 20.0 | 29.1 | 28.4 |
| Vanhatalo et al. (2008) | Finland | Separate | Finnish Ayrshire | EL | | 56 | 19.7 | 28.3 | 29.2 |
| Vanhatalo et al. (2009) | Finland | Separate | Finnish Ayrshire | 77 | | 61 | 20.4 | 27.1 | 26.5 |
| Weiss and Shockey (1991) | Ohio, US | TMR | Holstein | 140 | 82% forage | 82 | 19.2 | 22.5 | 21.0 |
| | | | | | 63% forage | 63 | 21.5 | 27.1 | 23.5 |
| | | | | | 43% forage | 43 | 22.5 | 27.2 | 23.7 |

[1] The number in brackets refers to experiment number within publication. [2] Days in milk. [3] Proportion of forage in the total ration on dry matter basis. [4] Dry matter intake, kg/day. [5] Milk yield, kg/day. [6] Energy corrected milk (3.14 MJ/kg), kg/day. [7] Early lactation. [8] Concentrate. [9] Not reported.

## 5.2 Paper II – Digestibility and clover proportion determine milk production when silages of different grass and clover species are fed to dairy cows

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# Digestibility and clover proportion determine milk production when silages of different grass and clover species are fed to dairy cows

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## ABSTRACT

This study examined how silages of different grass and clover species affect dry matter (DM) intake, milk production, and eating behavior in dairy cows. The primary growth of perennial ryegrass (early and late harvested), festulolium, tall fescue, red clover, and white clover swards were cut, wilted, and ensiled without additives. Thirty-six Danish Holstein cows were fed ad libitum with total mixed rations containing 70% forage on DM basis in an incomplete Latin square design. The forage source was either 1 of the 6 pure silages or late perennial ryegrass silage mixed (50:50 on DM basis) with either red clover or white clover silage. Intake of DM, milk yield, and milk lactose concentration were higher, whereas milk fat and protein concentrations were lower when cows were fed clover compared with grass. No differences in DM intake and milk composition were detected between cows fed red clover and white clover, but white clover resulted in higher milk yield than red clover. Lower body weight, probably caused by lower rumen fill, in cows fed pure white clover compared with the other treatments indicated that intake was regulated physiologically instead of physically. Cows fed early perennial ryegrass, which had the highest silage organic matter digestibility (OMD), did not produce the expected amount of energy-corrected milk (ECM) compared with the other treatments based on the amount of OM digested in the gastrointestinal tract, but the reason was unclear. Across all other grass species, ECM was related to OMD. Inclusion of 50% clover in the diet increased ECM with 2.3 kg/d, and the response to OMD was comparable to the response for the grass silages. In situ fiber degradation profile parameters indicated that fiber in festulolium differed compared with fiber in the other grass species and resembled fiber in clover. Drinking and eating behavior differed markedly in cows fed pure white clover compared with the other treatments. Water intake per drinking bout was comparable among treatments, but cows fed pure white clover had higher drinking bout duration and reduced drinking rate. Additionally, meal size was smaller for cows fed pure white clover compared with the other treatments, for which meal size was similar. In conclusion, differences in ECM between different grass species can be explained by differences in OMD, and at a given OMD level inclusion of clover in the diet increased ECM.
**Key words:** legume, organic matter digestibility, eating behavior, drinking behavior, feces score

## INTRODUCTION

Forages with high field yields are important to sustain a profitable milk production system; simultaneously, dairy cows require forages that are highly digestible to provide a high milk yield. Grass-clover silages often constitute a major part of the feed rations for dairy cows, and thus a stable production of high-quality herbage is essential. Yield and quality of different grass and legume species are highly dependent on geographical location, weather conditions, and farming management; therefore, selection of species is important for an optimal forage production. Alstrup et al. (2016) found that grass-clover silages of different cuts had similar values for milk production at comparable digestibility. Recently, a meta-analysis showed that milk production, in cows fed different grass species, is comparable provided that OM digestibility is similar (Johansen et al., 2017). However, few studies have compared different grass species. Furthermore, it is well documented that DMI and milk production generally are higher in cows fed legume-based diets compared with grass-based diets (Steinshamn, 2010; Johansen et al., 2017), as legumes contain less fiber than grasses and have a higher degradation rate of fiber in the rumen due to differences between legumes and grasses in the physical position of lignin (Buxton and Redfearn, 1997). However, effects of clover versus grass on eating behavior are scarce in the literature. Different grass and clover species vary in

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morphology, resulting in widely different leaf-to-stem ratios, which could affect eating behavior and milk production potential of single species.

The objective of the current study was to investigate how silages of the most relevant grass and clover species under Danish conditions affect DMI, eating behavior, and milk production in dairy cows. Our hypotheses were that (1) at similar silage OM digestibility, intake of clover silage is higher than intake of grass silage, resulting in a higher milk production and (2) milk production in cows fed silages of different grass or clover species reflects silage OM digestibility.

## MATERIALS AND METHODS

The current experiment complied with the guidelines of Danish Ministry of Environment and Food (2014) Law No. 474 (May 15, 2014) concerning animal experimentation and care of animals under study.

### *Fields and Harvest*

In the beginning of April 2014, fields with pure perennial ryegrass (*Lolium perenne* L. 'Calvano 1'), festulolium (*Festulolium braunii* K.A 'Perun'), tall fescue (*Festuca arundinacea* Schreb. 'Tower'), red clover (*Trifolium pratense* L. 'Suez'), and white clover (*Trifolium repens* L. 'Silvester') were established at AU Foulum (56°29′N, 9°35′E), Tjele, Aarhus University. These species were selected because perennial ryegrass is the dominating grass species in the oceanic climate of Europe, including Denmark; festulolium and tall fescue have been more common in Danish agriculture the last decade due to some advantages of cultivation compared with perennial ryegrass, and red clover and white clover are the major legume species in grass and pasture fields in Europe. Barley (*Hordeum vulgare* L. 'Columbus') was established as cover crop, with an amount of 110 kg/ha, and perennial ryegrass, festulolium, tall fescue, red clover, and white clover were sown with an amount of 21, 24, 36, 6.3, and 6.2 kg/ha, respectively. The cover crop was harvested in the middle of July, as whole crop on the clover fields, and in the beginning of August, at maturity on the grass fields. All fields were cut in the autumn 2014 to prepare the fields for winter. Before seeding, the fields received 25 t of liquid manure per hectare, and N and S liquid inorganic fertilizer was supplied as needed. After harvest of the cover crop, the fields with festulolium and tall fescue received 20, 3, 12, and 5 kg of liquid inorganic N, P, K, and S, respectively, to maintain growth. In the middle of March 2015, the grass fields received 118 kg of N, 16 kg of P, 47 kg of K, and 16 kg of S per hectare, and the clover fields received 116 kg of K/ha allocated as liquid inorganic fertilizer. The primary growth of all species was mown in 2015 with a disc mower conditioner set to 7 cm stubble height and wilted on broad swaths to achieve a DM concentration of approximately 350 g/kg. The plan was to mow perennial ryegrass at 2 time points to obtain variation in OM digestibility, and the aim was that OM digestibility of tall fescue and festulolium should be within the range of the 2 perennial ryegrass cuts. Tall fescue, festulolium, and half of the perennial ryegrass (early perennial ryegrass) were mown May 21 and wilted for 3 d because of rain (3.2 mm) in the middle of the wilting period. The remaining perennial ryegrass (late perennial ryegrass), red clover, and white clover were all mown June 3 and wilted for 2 d. The weed contamination was estimated visually to be below 2% in all fields. The developmental stage (Skinner and Moore, 2007) at harvest was elongation stage with 1, 2, and 3 nodes noticeable or visible for tall fescue, early perennial ryegrass, and festulolium, respectively, reproductive stage with visible spikelets for late perennial ryegrass, and late vegetative stage for red clover. White clover was in a vegetative stage without buds. After wilting, the crops were raked and chopped with a theoretical length of 15 mm. The chopped crops were unloaded on a clean concrete area and mixed within crop to ensure homogeneity before baling in a stationary round baler (Orkel MP 2000, Orkel A/S, Fannrem, Norway). The bales were wrapped with 12 layers of plastic and ensiled without additives.

Prior to mowing, 5 spots (30 × 30 cm) were randomly selected in each field, cut with shears to 7 cm stubble height, and pooled within species. To determine the leaf-to-stem ratio on DM basis, leaves (leaf blade and petiole) and stems (leaf sheath, stem, and flower) were separated by hand and dried for 48 h at 60°C.

### *Animals, Feeding, Housing, and Sampling*

Thirty-six Danish Holstein cows, 12 primiparous and 24 multiparous, were used for the experiment. The primiparous cows were 59 ± 12 (mean ± SD) DIM and the multiparous cows were 95 ± 64 DIM at the beginning of the experiment. All cows were fed ad libitum with a TMR based on 70% forage (DM basis), divided into 2 equal daily meals fed at 0800 and 1600 h. The amount fed was adjusted daily to achieve 7 to 10% leftovers, but a minimum of 3 kg. The only difference between treatments (Table 1) was the forage source, which was silage of either (1) early perennial ryegrass (**EPR**), (2) festulolium (**FEST**), (3) tall fescue (**TF**), (4) late perennial ryegrass (**LPR**), (5) 50% red clover:50% late perennial ryegrass (**RC-LPR**), (6) 50% white clover:50 % late perennial ryegrass (**WC-LPR**), (7) red clover (**RC**), or (8) white clover (**WC**). The TMR were mixed

daily before the morning feeding for 12 min in a Cormall auger mixer (Cormall A/S, Sønderborg, Denmark). The concentrate part consisted mainly of soybean meal and rolled wheat (Table 1) to reduce the amount of fiber originating from the concentrate. The LPR ration was adjusted with urea to reach a CP concentration of 160 g/kg of DM. Titanium oxide ($TiO_2$) was included in the TMR as external marker to estimate fecal output.

Multiparous cows were blocked according to DIM and randomly assigned to 1 of the 8 dietary treatments in an 8 × 8 incomplete Latin square design with 4 periods. The 8 primiparous cows latest in lactation were randomly assigned to 1 of the 8 dietary treatments in an 8 × 8 incomplete Latin square design, similar to the multiparous cows, whereas the 4 primiparous cows earliest in lactation were randomly assigned to 1 of the 4 pure grass treatments (treatment 1–4) in a 4 × 4 Latin square design. Each period lasted 21 d. The experimental design resulted in 20 observations for the 4 grass treatments (treatment 1–4) and 16 observations for the 4 treatments including clover (treatment 5–8).

The cows were housed in a loose-housing system with concrete floor and cubicles with mattresses and sawdust as bedding in 2 groups according to parity (primiparous and multiparous). During the whole experiment, each cow had access to its own feeding trough, where an electronic ear tag controlled opening of the gate. One drinking trough per 6 cows was available for ad libitum intake of water, and all cows had access to all drinking troughs within group. The water troughs were filled up with approximately 36 L between visits. Feed and water intake along with number and duration of visits were measured automatically with the Insentec RIC system (Insentec, Marknesse, the Netherlands). The cows were milked twice daily at 0545 and 1645 h in a milking parlor. Milk yield was registered daily and milk samples were taken over 3 d (6 milkings) in the last week of each period and analyzed for fat, protein, and lactose monohydrate (Eurofins Steins Laboratorium, Vejen, Denmark). In the milking parlor exit, a platform scale was installed and live weight of the cows was measured automatically twice a day.

Six subsamples of feces (350 mL) were collected during the last 4 d of each period (d 18–20 at 1400 h and d 19–21 at 0800 h) and frozen immediately after collection. At the end of the experimental period, fecal samples were thawed and pooled within cow and period. Feces consistency was scored on a 5-point (1–5; 1 is loose and 5 is firm) visual observation scale, using half points, before drying at 60°C for 72 h for DM determination and chemical analysis.

Each silage bale, when opened, was sampled by taking 5 to 10 subsamples randomly with the hand at different places in the bale to make a representative sample; a subsample was used for DM determination and another subsample was pooled within period. Samples of soybean meal and rolled wheat were taken once a week and pooled within period. Before drying and chemical analyses, period 1 and 2 and period 3 and 4 were pooled for both silages and concentrates. Furthermore, a silage sample pooled over all 4 periods was used to analyze carbohydrate fractions and for in situ studies of NDF and CP degradation. Samples of the TMR were taken daily the last 4 d of each period and DM concentration was determined and used to calculate DMI. A pooled sample within period was dried (60°C) and used for chemical analyses.

### Chemical Analyses

Samples for chemical analyses were ground to 1 mm (ZM 200 mill, Retsch GmbH, Haan, Germany). Ash concentration was determined in all samples by combustion at 525°C for 6 h. Nitrogen was determined using a Vario MAX CN (Elementar Analysesysteme GmbH, Hanau, Germany) following the Dumas method (Hansen, 1989) and multiplied by 6.25 to determine CP. Following the Ankom procedures (Ankom, 2016), NDF, ADF, and ADL were determined sequentially according to Mertens (2002) using heat-stable amylase for the NDF step; values were corrected for ash using the ADL ash residue.

Titanium oxide in TMR and fecal samples was measured by digestion of samples with sulfuric acid and measuring of absorbance after addition of hydrogen peroxide (Myers et al., 2004). In feedstuffs, crude fat was analyzed after HCl hydrolysis and petroleum ether extraction (Stoldt, 1952) using a Soxtec system (Foss Analytical, Hillerød, Denmark). Total sugar was determined by the Luff-Schoorl method (European Community, 2012, 71/250/EEC). Soluble N in silages was determined by one-hour extraction (39°C) in a borate-phosphate buffer (pH 6.75; Åkerlind et al., 2011). Silage samples were incubated in rumen fluid for 48 h, followed by incubation in a pepsin-HCl solution according to Tilley and Terry (1963), and residues were combusted to determine in vitro OM digestibility (% of OM). Silage in vivo OM digestibility (**OMD**; % of OM) was calculated as 4.10 + 0.959 × in vitro OM digestibility (Møller et al., 1989; Åkerlind et al., 2011). In concentrate samples, the enzymatic digestibility of OM (% of OM) was determined and OMD was calculated as 5.38 + 0.867 × enzymatic digestibility of OM (Weisbjerg and Hvelplund, 1993; Åkerlind et al., 2011).

To measure silage pH and fermentation products, extracts were prepared by blending 100 g of chopped silage with 1 L of water, followed by centrifugation (2,300 × *g*, 20 min, 10°C). The pH was measured in

**Table 1**. Ration ingredients and chemical composition (g/kg of DM, unless otherwise stated) for the 8 dietary treatments

| | Treatment[1] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Item | EPR | FEST | TF | LPR | RC-LPR | WC-LPR | RC | WC |
| Ingredient | | | | | | | | |
| Early perennial ryegrass silage | 700 | | | | | | | |
| Festulolium silage | | 700 | | | | | | |
| Tall fescue silage | | | 700 | | | | | |
| Late perennial ryegrass silage | | | | 696 | 350 | 350 | | |
| Red clover silage | | | | | 350 | | 700 | |
| White clover silage | | | | | | 350 | | 700 |
| Soybean meal | 120 | 120 | 120 | 119 | 120 | 120 | 120 | 120 |
| Wheat, rolled | 158 | 158 | 158 | 157 | 158 | 158 | 158 | 158 |
| Mineral and vitamin mix[2] | 18.5 | 18.5 | 18.5 | 18.4 | 18.5 | 18.5 | 18.5 | 18.5 |
| NaCl | 2.2 | 2.2 | 2.2 | 2.2 | 2.2 | 2.2 | 2.2 | 2.2 |
| Urea, 46% N | | | | 6.2 | | | | |
| Titanium oxide | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 |
| Chemical composition[3] | | | | | | | | |
| DM, g/kg of fresh matter | 432 ± 2.8 | 372 ± 1.1 | 499 ± 2.5 | 425 ± 2.3 | 401 ± 1.7 | 404 ± 1.1 | 381 ± 1.4 | 386 ± 1.9 |
| Ash | 81.0 ± 0.6 | 87.1 ± 0.4 | 85.9 ± 0.6 | 74.5 ± 0.2 | 90.6 ± 1.2 | 93.0 ± 1.3 | 107 ± 0.7 | 109 ± 1.7 |
| CP | 171 ± 1.6 | 167 ± 2.3 | 178 ± 3.1 | 159 ± 1.3 | 181 ± 2.7 | 206 ± 1.7 | 213 ± 2.1 | 268 ± 2.3 |
| NDF | 316 ± 1.7 | 340 ± 3.1 | 372 ± 4.6 | 369 ± 1.9 | 315 ± 3.1 | 283 ± 5.8 | 248 ± 3.5 | 202 ± 3.9 |
| ADF | 164 ± 1.2 | 183 ± 1.6 | 187 ± 2.3 | 199 ± 1.2 | 182 ± 0.9 | 163 ± 3.1 | 160 ± 1.5 | 131 ± 1.1 |

[1]EPR = early perennial ryegrass; FEST = festulolium; TF = tall fescue; LPR = late perennial ryegrass; RC-LPR = 50% red clover:50% late perennial ryegrass; WC-LPR = 50% white clover:50% late perennial ryegrass; RC = red clover; WC = white clover.

[2]Composition: Ca, 142 g/kg; P, 44 g/kg; Mg, 58 g/kg; Na, 80 g/kg; Mn, 3,546 mg/kg; Zn, 3,989 mg/kg; Cu, 1,330 mg/kg; Co, 22 mg/kg; I, 199 mg/kg; Se, 47 mg/kg; vitamin $K_3$, 99 mg/kg; vitamin A, 883 IU/g; vitamin $D_3$, 182 IU/g; vitamin E, 6.4 IU/g.

[3]n = 16 for DM and n = 4 for the remaining components. Mean ± SEM is given.

the supernatant. In silage extracts stabilized with 5% meta-phosphoric acid, VFA was analyzed by GC according to Kristensen et al. (1996). Ammonia N was analyzed using a kit based on glutamate dehydrogenase (AM 1015; Randox Laboratories Ltd., Crumlin, UK) and a Cobas Mira auto-analyzer (Triolab A/S, Brøndby, Denmark). Glucose was analyzed using an YSI 2900 Biochemistry Analyzer (YSI Inc., Yellow Springs, OH) and membrane-immobilized substrate specific oxidases. Alcohols and alcohol esters were analyzed by headspace GC-MS (Kristensen et al., 2010). In unstabilized silage extracts, DL-lactate was analyzed by GC-MS (Kristensen et al., 2010).

In silage samples pooled over all 4 periods, water-soluble carbohydrates were determined colorimetrically using the phenol-sulfuric acid assay (DuBois et al., 1956). Starch was analyzed according to the acetate buffer method described by Hall (2009), with the modifications that samples were incubated at 50°C instead of 60°C, 3.0 mL of glucose oxidase-peroxidase reagent was used instead of 2.5 mL, and the last incubation was done at 50°C for 20 min. Neutral detergent soluble fiber (**NDSF**) was determined both by extracting samples in 80% ethanol for 4 h at 20°C and by extracting samples in water for 1 h at 40°C. In the ethanol or water insoluble residues, OM (IROM), and CP (IRCP) were determined and NDSF was calculated as IROM – IRCP – NDF – starch (Hall et al., 1999). Silage samples [1.5 mm milling in Dacron (TP Filter, Upplands Väsby, Sweden) bags with 12 μm pore size] were incubated in the rumen of dry cows fed at maintenance for 288 h and indigestible NDF (**iNDF**) was determined as the remaining NDF residue (Åkerlind et al., 2011). To determine in situ rumen degradation kinetics of CP and NDF, silage samples (1.5 mm milling in Dacron bags with 38 μm pore size) were incubated for 0, 2, 4, 8, 16, 24, 48, and 96 h, and further for 168 h for NDF in the rumen of dry cows fed at maintenance. Degradation profile parameters for CP were estimated according to the equation of Ørskov and McDonald (1979) and corrected for particle loss, estimated as the difference between 0 h solubility and solubility over filter paper (Hvelplund and Weisbjerg, 2000). Degradation profile parameters and lag time for NDF were estimated according to McDonald (1981) without the soluble fraction, as NDF is insoluble per definition, and the values used for parameter estimation were corrected for possible initial particle loss by relating the NDF residues to the NDF residue at 0 h of incubation, where bags were only washed. Disappearance of CP in the gastrointestinal tract was estimated in situ using the mobile bag technique described by Hvelplund et al. (1992), where silage samples (1.5 mm milling in nylon bags with 11 μm pore size) were incubated for 16 h in the rumen of dry cows fed at maintenance, followed by incubation in a pepsin-HCl solution (pH 2.4). Finally, the bags were inserted into the small intestine through the duodenal cannula of lactating cows and collected in feces. After washing, residues of both rumen and mobile bag incubations were transferred to N-free filter paper, and N residues were measured quantitatively using the Kjeldahl procedure.

### Calculations

Energy-corrected milk yield (3.14 MJ/kg) was calculated according to Sjaunja et al. (1991) using the formula ECM = 0.01 × milk yield (kg) + 12.2 × milk fat (kg) + 7.7 × milk protein (kg) + 5.3 × milk lactose (kg), where lactose was measured as lactose monohydrate and averaged over the last 3 d in each period. Dry matter intake was averaged over the last 4 d in each period. Titanium oxide was used as digestion marker and concentrations in TMR and feces were used to calculate fecal DM output and apparent total-tract digestibility of nutrients. Supply of $NE_L$ was calculated on individual level according to the Nordic feed evaluation system (NorFor) described by Volden and Nielsen (2011). Live weight of cows was averaged over the last 10 d of each period, but the last 5 d in period 3 were discarded, as the weight was unstable due to freezing weather.

In eating behavior data, initiation of a new meal was defined if the time between termination of one visit to onset of a new visit in the feeding trough was above 8 min (Dürst et al., 1993). Initiation of a new drinking bout was defined if the time between termination of one visit to onset of a new visit to a drinking trough was above 4 min (Dado and Allen, 1994). Eating and drinking duration, both total and for each meal and drinking bout, was equal to the time the gate to the feeding or drinking trough was opened.

### Statistical Analyses

Statistical analyses were conducted in R 3.3.2 (R Core Team, 2016). The effect of treatment on the various animal responses was analyzed with the following linear mixed model fitted with REML and the lmer function from the lme4 package (Bates et al., 2015):

$$Y_{tlpdc} = \mu + \alpha_t + \beta_l + \gamma_p + \delta d + (\beta\gamma)_{lp} + A_c + E_{tlpdc},$$

where $Y_{tlpdc}$ is the dependent response variable, $\mu$ is the overall mean, $\alpha$ is the fixed effect of treatment ($t$ = EPR, FEST, TF, LPR, RC-LPR, WC-LPR, RC, WR),

β is the fixed effect of parity ($l$ = primiparous, multiparous), γ is the fixed effect of period (p = 1 to 4), δ is the regression coefficient for DIM $d$, $(\beta\gamma)_{lp}$ is the interaction between parity and period, $A$ is the random effect of cow ($c$ = 1 to 36), and $E_{tlpdc}$ is the random residual error assumed to be independent with constant variance and normally distributed. The remaining 2-way interactions were tested as well, but none were significant or improved the model. One cow in 1 period was discarded in the analysis because of a feeding mistake.

Least squares means (**LSM**) and standard error of mean, obtained using the lsmeans package (Lenth, 2016), are presented in the tables. Differences between LSM were evaluated using Tukey's method for comparing a family of 8 estimates. The contrast function was used to test the general effect of grass against clover (EPR, FEST, TF, and LPR vs. RC and WC), the general effect of red clover against white clover (RC-LPR and RC vs. WC-LPR and WC), and linear and quadratic effects of increasing the proportion of red clover and white clover, respectively, using LPR as the treatment without clover. Some response variables related to water intake and eating and drinking behavior were $\log_{10}$ or inverse transformed to obtain variance homogeneity and normality of residuals. For these variables, the transformed data with the statistics are presented in the table along with the back-transformed LSM. Statistical significance was regarded by $P$-values $\leq 0.05$ and tendencies were considered by $P$-values $\leq 0.10$.

## RESULTS

### Silages and TMR

Table 2 shows the chemical composition of used feedstuffs. Silage DM concentration ranged from 295 g/kg in festulolium silage to 414 g/kg in tall fescue silage. The clover silages generally had higher ash and CP concentrations and a lower NDF concentration compared with the grass silages. All silages reached a low pH (4.16–4.45) during ensiling, but the concentration of lactate was higher in clover silages than in grass silages (117–133 vs. 59.7–79.4 g/kg of DM). For the remaining fermentation products, no general differences between grass and clover silages were observed. Red clover silage had lower concentrations of soluble N and ammonia N as a proportion of total N compared with the other silages (49.3 vs. 61.8–70.7% and 6.80 vs. 7.40–8.27%, respectively). The postponed harvest of perennial ryegrass resulted in lower ash, CP, and crude fat concentrations and a higher NDF concentration. The concentration of NDSF was higher in clover silages compared with grass silages when extracted with both water and ethanol (Table 3). The iNDF as proportion of NDF was higher in clover silages compared with grass silages (18.2–23.6 vs. 7.74–11.9%), which also was evident from the rumen degradable fraction of NDF being lower in clover silages than in grass silages. The degradation rate of the rumen-degradable fraction was higher in the clover silages than in the grass silages for both NDF and CP (Table 3). Silage OMD varied from 73.9% in tall fescue silage to 83.4% in early perennial ryegrass silage (Table 2), and OMD in the clover silages was within the range covered by the grass silages. Variation in chemical composition between TMR (Table 1) reflected the variation in chemical composition between silages, except CP in LPR, as we added urea to this ration to achieve a CP concentration of 160 g/kg of DM, which was obtained (159 g of CP/kg of DM; Table1).

### Intake and Weight

Intake of DM varied from 18.8 to 21.7 kg/d and was higher in cows fed clover than in cows fed grass ($P < 0.01$), with no difference in DMI between cows fed red clover or white clover ($P = 0.23$; Table 4). The treatments EPR and FEST resulted in higher DMI than TF and LPR ($P < 0.05$). The DMI increased linearly when increasing the red clover proportion, whereas increasing the white clover proportion resulted in a quadratic effect, as DMI was similar for WC-LPR and WC. Nutrient intake reflected DMI and chemical composition of the TMR. Cows on the WC treatment were on average 16 kg lighter than cows on the other treatments ($P < 0.05$; Table 4).

### Milk Yield and Composition

Cows fed clover had a higher milk yield than cows fed grass (33.8 vs. 29.4 kg/d; $P < 0.01$), and cows fed white clover had a higher milk yield than cows fed red clover (33.7 vs. 32.2 kg/d; $P < 0.01$; Table 4). The FEST treatment resulted in higher milk yield than TF and LPR. Milk fat and milk protein concentrations were lower (41.8 vs. 45.9 and 34.1 vs. 36.0 g/kg, respectively; $P < 0.01$), whereas milk lactose concentration was higher (48.4 vs. 47.1 g/kg; $P < 0.01$) when cows were fed clover compared with grass. Milk composition did not differ between cows fed red clover and white clover, but increasing clover proportion resulted in linear effects for all milk composition parameters for both red clover and white clover. Total daily fat production was unaffected of red clover proportion, whereas white clover resulted in a quadratic effect, with the highest daily fat production on the WC-LPR treatment. The LPR increased the milk fat concentration (47.0

**Table 2**. Chemical composition[1] (mean ± SEM) of used feedstuffs[2] (g/kg of DM, unless otherwise stated)

| | Silage[3] | | | | | | Concentrates | |
|---|---|---|---|---|---|---|---|---|
| Item | EPR | FEST | TF | LPR | RC | WC | Soybean meal | Wheat, rolled |
| Cutting date | May 21 | May 21 | May 21 | June 3 | June 3 | June 3 | | |
| Leaf:stem ratio | 57:43 | 45:55 | 80:20 | 37:63 | 43:57 | 100:0 | | |
| DM, g/kg of fresh matter | 350 ± 2.1 | 295 ± 1.9 | 414 ± 2.2 | 350 ± 1.6 | 301 ± 1.9 | 304 ± 1.3 | 899 ± 0.4 | 885 ± 0.5 |
| Ash | 74.4 ± 0.70 | 82.7 ± 0.50 | 81.9 ± 0.20 | 64.0 ± 0.15 | 113 ± 1.1 | 116 ± 1.0 | 66.7 ± 1.40 | 24.5 ± 1.80 |
| CP | 130 ± 0.9 | 126 ± 0.6 | 140 ± 0.6 | 93.4 ± 0.31 | 188 ± 1.3 | 267 ± 1.6 | 479 ± 9.7 | 165 ± 7.8 |
| NDF | 398 ± 2.2 | 426 ± 1.4 | 482 ± 2.3 | 461 ± 2.8 | 310 ± 5.3 | 233 ± 3.8 | 98.4 ± 0.32 | 117 ± 1.0 |
| ADF | 216 ± 1.1 | 237 ± 3.8 | 252 ± 0.7 | 256 ± 1.6 | 212 ± 2.4 | 172 ± 0.8 | 35.6 ± 3.04 | 30.4 ± 1.01 |
| ADL | 4.95 ± 0.30 | 5.22 ± 0.99 | 6.31 ± 0.60 | 5.58 ± 0.42 | 24.0 ± 0.52 | 23.2 ± 1.69 | | |
| Crude fat | 28.8 ± 0.65 | 24.8 ± 0.00 | 23.9 ± 0.20 | 20.6 ± 0.50 | 24.1 ± 0.80 | 34.4 ± 0.35 | 17.8 ± 0.20 | 25.6 ± 0.45 |
| Total sugar | 89.6 ± 1.65 | 50.2 ± 3.50 | 14.5 ± 0.75 | 102 ± 0.2 | 34.7 ± 0.40 | 13.1 ± 1.60 | 107 ± 6.7 | 49.1 ± 2.05 |
| Glucose | 21.3 ± 0.30 | 11.2 ± 0.43 | 7.67 ± 0.06 | 14.4 ± 0.64 | 19.9 ± 1.58 | 0.97 ± 0.29 | | |
| N, % of total N | | | | | | | | |
| Soluble N | 70.7 ± 0.75 | 62.2 ± 0.81 | 64.5 ± 0.51 | 64.2 ± 1.79 | 49.3 ± 2.00 | 61.8 ± 3.04 | | |
| Ammonia N | 7.71 ± 0.04 | 8.27 ± 0.03 | 7.72 ± 0.08 | 7.44 ± 0.28 | 6.80 ± 0.17 | 7.40 ± 0.07 | | |
| OMD[4], % | 83.4 ± 0.24 | 80.6 ± 0.39 | 73.9 ± 0.17 | 77.0 ± 0.12 | 75.4 ± 0.04 | 82.2 ± 0.49 | 91.4 ± 0.04 | 89.6 ± 0.22 |
| pH | 4.18 ± 0.00 | 4.16 ± 0.01 | 4.45 ± 0.04 | 4.16 ± 0.02 | 4.23 ± 0.01 | 4.17 ± 0.02 | | |
| Lactate | 66.2 ± 0.91 | 79.4 ± 1.71 | 67.0 ± 0.29 | 59.7 ± 1.14 | 117 ± 3.5 | 133 ± 5.9 | | |
| Acetate | 24.1 ± 0.54 | 27.3 ± 0.11 | 22.7 ± 0.18 | 23.2 ± 0.02 | 29.0 ± 0.21 | 31.9 ± 0.56 | | |
| Caproate, mg/kg of DM | ND[5] | ND | ND | ND | 124 ± 2.57 | 56.5 ± 56.5 | | |
| Ethanol | 5.60 ± 0.28 | 15.2 ± 0.23 | 3.51 ± 0.06 | 16.2 ± 0.03 | 4.27 ± 0.49 | 4.29 ± 0.34 | | |
| Propanol, mg/kg of DM | ND | 9.87 ± 9.87 | 27.6 ± 27.6 | 19.9 ± 9.15 | ND | ND | | |
| Butanol, mg/kg of DM | 22.5 ± 0.34 | 12.3 ± 4.78 | 14.1 ± 0.66 | 39.1 ± 4.09 | 5.67 ± 1.05 | 611 ± 18.7 | | |
| Ethyl acetate, mg/kg of DM | 286 ± 11.8 | 698 ± 11.6 | 244 ± 5.4 | 684 ± 11.3 | 264 ± 45.1 | 309 ± 26.2 | | |
| Propyl acetate, mg/kg of DM | 2.64 ± 0.42 | 4.17 ± 1.67 | 6.18 ± 1.70 | 5.24 ± 0.31 | 5.25 ± 1.84 | 5.52 ± 0.11 | | |

[1]Silages were also analyzed for propionate and butyrate, but they were not detected. Propionate <1.5 g/kg of DM and butyrate <0.8 g/kg of DM.

[2]n = 2 except for silage DM, where n is 28, 28, 28, 47, 31, and 31 for EPR, FEST, TF, LPR, RC, and WC, respectively.

[3]EPR = early perennial ryegrass; FEST = festulolium; TF = tall fescue; LPR = late perennial ryegrass; RC = red clover; WC = white clover.

[4]In vivo OM digestibility calculated as 4.10 + 0.959 × in vitro OM digestibility (%) for silages (Møller et al., 1989) and as 5.38 + 0.867 × enzymatic digestibility of OM (%) for concentrates (Weisbjerg and Hvelplund, 1993).

[5]ND = not detected.

vs. 44.6 g/kg; $P < 0.05$) but reduced the milk protein concentration (35.7 vs. 36.8 g/kg; $P < 0.05$) compared with EPR, whereas FEST and TF did not differ from LPR. None of the grass treatments differed in milk lactose concentration. The 4 grass treatments resulted in similar ECM per kilogram of DMI, but the energy output in milk related to energy intake was lower for the EPR treatment than for the other grass treatments and kilograms of ECM per kilogram OM digested was lower for EPR than for FEST. Increasing the red clover proportion linearly decreased kilograms of ECM per kilogram of DMI, whereas white clover proportion did not affect the ECM production per kilogram of DMI. The energy output in milk related to energy intake did not differ between red clover and white clover.

### Feces

Fecal DM concentration was higher when cows were fed clover than grass (109 vs. 91 g/kg of fresh matter; $P < 0.01$), and red clover resulted in a higher fecal DM concentration than white clover (109 vs. 100 g/kg of fresh matter; $P < 0.01$; Table 5). Both red clover and white clover increased fecal DM concentration linearly. Feces texture for WC-LPR and WC was more liquid than for all grass treatments, even though fecal DM concentration was similar or higher. The relationship between fecal DM concentration and feces texture within treatment (Figure 1) was positive for all treatments, but the WC-LPR and WC treatments displaced the lines parallel to the left, giving more liquid feces even though DM concentrations were comparable with the other treatments. The chemical composition of feces reflected the chemical composition of the TMR, as feces from cows fed clover had a higher concentration of ash and CP and a lower concentration of NDF.

### Total-Tract Digestibility

The apparent total-tract digestibility of DM, OM, NDF, and ADF was higher for EPR than for FEST, TF, and LPR, which did not differ (Table 5). The FEST treatment resulted in a lower apparent total-tract CP digestibility than the other grass treatments, whereas the WC treatment resulted in a higher CP digestibility than all other treatments. Inclusion of red clover reduced DM, OM, and ADF digestibility linearly, whereas we observed a tendency for a quadratic decrease for NDF digestibility, as NDF digestibility for RC was lower than for RC-LPR and LPR (60.7 vs. 68.2–70.8%; Table 5). Inclusion of white clover did not affect DM, OM, NDF, or ADF digestibility. The linear and quadratic effects of clover proportion on CP digestibility were probably affected by urea addition to LPR, and therefore will not be examined further.

**Table 3**. Carbohydrate fractions, NDF, and CP rumen degradation parameters, and disappearance of CP from mobile bags for used silages (n = 1)

| | Silage[1] | | | | | |
|---|---|---|---|---|---|---|
| Item | EPR | FEST | TF | LPR | RC | WC |
| WSC,[2] g/kg of DM | 130 | 84.2 | 42.8 | 127 | 71.7 | 28.4 |
| Starch, g/kg of DM | 1.36 | 1.02 | 0.81 | 0.86 | 6.93 | 0.93 |
| NDSF ethanol,[3] g/kg of DM | 97.8 | 105 | 83.3 | 120 | 156 | 162 |
| NDSF water,[4] g/kg of DM | 87.9 | 90.5 | 79.6 | 95.0 | 132 | 142 |
| iNDF,[5] g/kg of NDF | 77.4 | 101 | 103 | 119 | 236 | 182 |
| NDF rumen degradation | | | | | | |
| b,[6] g/kg of NDF | 936 | 902 | 907 | 891 | 747 | 820 |
| c,[7] %/h | 4.54 | 5.12 | 4.06 | 4.00 | 7.79 | 8.03 |
| Lag time, h | 1.37 | 1.24 | 1.22 | 1.72 | 1.18 | 1.99 |
| CP rumen degradation | | | | | | |
| Particle loss, g/kg of CP | 81.0 | 96.9 | 101 | 102 | 87.0 | 91.1 |
| a,[8] g/kg of CP | 686 | 604 | 611 | 631 | 482 | 598 |
| b, g/kg of CP | 256 | 327 | 315 | 258 | 477 | 372 |
| c, %/h | 6.11 | 6.23 | 6.59 | 5.18 | 14.4 | 13.4 |
| Disappearance of CP from mobile bags, % | 89.2 | 86.0 | 88.0 | 86.0 | 91.8 | 94.7 |

[1]EPR = early perennial ryegrass; FEST = festulolium; TF = tall fescue; LPR = late perennial ryegrass; RC = red clover; WC = white clover.
[2]Water-soluble carbohydrates.
[3]Neutral detergent soluble fiber determined using ethanol as solvent.
[4]Neutral detergent soluble fiber determined using water as solvent.
[5]Indigestible NDF determined by 288 h of in situ incubation.
[6]Insoluble, but rumen-degradable fraction.
[7]Fractional rate of degradation of fraction b.
[8]Rumen-soluble fraction.

**Table 4**. Effect of forage source on intake, milk yield, milk composition, and cow weight

| | Treatment[1] | | | | | | | | | Contrast[3] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | EPR | FEST | TF | LPR | RC-LPR | WC-LPR | RC | WC | SEM[2] | G.C | R.W | RC.L | RC.Q | WC.L | WC.Q |
| No. of cows | 19 | 20 | 20 | 20 | 16 | 16 | 16 | 16 | | | | | | | |
| Intake, kg/d | | | | | | | | | | | | | | | |
| DM | 20.2$^{b}$ | 20.3$^{b}$ | 18.8$^{c}$ | 19.1$^{c}$ | 20.8$^{ab}$ | 21.5$^{a}$ | 21.7$^{a}$ | 21.6$^{a}$ | 0.33 | <0.01 | 0.23 | <0.01 | 0.15 | <0.01 | <0.01 |
| CP | 3.43$^{d}$ | 3.37$^{d}$ | 3.35$^{d}$ | 3.04$^{e}$ | 3.76$^{c}$ | 4.46$^{b}$ | 4.65$^{b}$ | 5.91$^{a}$ | 0.068 | <0.01 | <0.01 | <0.01 | 0.22 | <0.01 | 0.82 |
| NDF | 6.37$^{cd}$ | 6.92$^{ab}$ | 7.00$^{a}$ | 7.04$^{a}$ | 6.57$^{bc}$ | 6.04$^{d}$ | 5.19$^{e}$ | 4.23$^{f}$ | 0.116 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| ADF | 3.30$^{c}$ | 3.72$^{a}$ | 3.52$^{b}$ | 3.80$^{a}$ | 3.79$^{a}$ | 3.51$^{b}$ | 3.46$^{bc}$ | 2.77$^{d}$ | 0.062 | <0.01 | <0.01 | <0.01 | 0.01 | <0.01 | <0.01 |
| $NE_L$, MJ/d | 140$^{bc}$ | 134$^{d}$ | 117$^{f}$ | 125$^{e}$ | 134$^{cd}$ | 143$^{ab}$ | 138$^{bcd}$ | 148$^{a}$ | 2.0 | <0.01 | <0.01 | <0.01 | 0.14 | <0.01 | <0.01 |
| Yield, kg/d | | | | | | | | | | | | | | | |
| Milk | 30.2$^{cd}$ | 30.8$^{c}$ | 27.9$^{e}$ | 28.7$^{de}$ | 31.2$^{bc}$ | 32.8$^{ab}$ | 33.1$^{a}$ | 34.6$^{a}$ | 0.78 | <0.01 | <0.01 | <0.01 | 0.45 | <0.01 | 0.02 |
| ECM | 32.4$^{cd}$ | 33.7$^{bc}$ | 29.9$^{e}$ | 31.4$^{de}$ | 33.3$^{c}$ | 35.4$^{ab}$ | 33.8$^{bc}$ | 35.8$^{a}$ | 0.65 | <0.01 | <0.01 | <0.01 | 0.20 | <0.01 | <0.01 |
| Fat | 1.33$^{de}$ | 1.42$^{abc}$ | 1.25$^{e}$ | 1.34$^{cd}$ | 1.38$^{bcd}$ | 1.49$^{a}$ | 1.36$^{bcd}$ | 1.44$^{ab}$ | 0.029 | <0.01 | <0.01 | 0.58 | 0.20 | <0.01 | <0.01 |
| Protein | 1.10$^{bc}$ | 1.10$^{bc}$ | 0.98$^{d}$ | 1.02$^{d}$ | 1.09$^{c}$ | 1.15$^{ab}$ | 1.11$^{bc}$ | 1.18$^{a}$ | 0.022 | <0.01 | <0.01 | <0.01 | 0.17 | <0.01 | <0.01 |
| Lactose | 1.42$^{de}$ | 1.45$^{d}$ | 1.32$^{f}$ | 1.35$^{ef}$ | 1.49$^{cd}$ | 1.55$^{bc}$ | 1.61$^{ab}$ | 1.67$^{a}$ | 0.038 | <0.01 | <0.01 | <0.01 | 0.71 | <0.01 | 0.09 |
| Milk composition, g/kg | | | | | | | | | | | | | | | |
| Fat | 44.6$^{b}$ | 46.8$^{a}$ | 45.4$^{ab}$ | 47.0$^{a}$ | 45.1$^{ab}$ | 45.5$^{ab}$ | 41.5$^{c}$ | 42.1$^{c}$ | 0.86 | <0.01 | 0.33 | <0.01 | 0.17 | <0.01 | 0.12 |
| Protein | 36.8$^{a}$ | 36.1$^{ab}$ | 35.4$^{bc}$ | 35.7$^{b}$ | 35.3$^{bc}$ | 35.4$^{bc}$ | 33.8$^{d}$ | 34.4$^{cd}$ | 0.54 | <0.01 | 0.18 | <0.01 | 0.10 | <0.01 | 0.36 |
| Lactose | 47.1$^{c}$ | 47.0$^{c}$ | 47.3$^{c}$ | 46.9$^{c}$ | 47.5$^{bc}$ | 47.3$^{c}$ | 48.6$^{a}$ | 48.2$^{ab}$ | 0.25 | <0.01 | 0.10 | <0.01 | 0.33 | <0.01 | 0.28 |
| kg of ECM/kg of DMI | 1.61$^{ab}$ | 1.66$^{a}$ | 1.60$^{ab}$ | 1.65$^{a}$ | 1.60$^{ab}$ | 1.65$^{a}$ | 1.57$^{b}$ | 1.66$^{a}$ | 0.027 | 0.26 | <0.01 | <0.01 | 0.70 | 0.86 | 0.97 |
| MJ of milk/$NE_L$ of intake | 0.73$^{c}$ | 0.79$^{ab}$ | 0.81$^{a}$ | 0.79$^{ab}$ | 0.78$^{ab}$ | 0.78$^{ab}$ | 0.77$^{ab}$ | 0.76$^{bc}$ | 0.013 | 0.05 | 0.41 | 0.07 | 0.81 | <0.01 | 0.77 |
| kg of ECM/kg of OM digested | 2.18$^{b}$ | 2.36$^{a}$ | 2.28$^{ab}$ | 2.31$^{ab}$ | 2.32$^{a}$ | 2.37$^{a}$ | 2.33$^{a}$ | 2.37$^{a}$ | 0.039 | 0.02 | 0.18 | 0.66 | 0.91 | 0.18 | 0.41 |
| Cow weight, kg | 619$^{a}$ | 622$^{a}$ | 618$^{a}$ | 618$^{a}$ | 625$^{a}$ | 621$^{a}$ | 618$^{a}$ | 605$^{b}$ | 9.4 | <0.01 | <0.01 | 0.93 | 0.01 | <0.01 | <0.01 |

$^{a–f}$Values within same line with different superscripts differ ($P < 0.05$).

[1]EPR = early perennial ryegrass; FEST = festulolium; TF = tall fescue; LPR = late perennial ryegrass; RC-LPR = 50% red clover:50% late perennial ryegrass; WC-LPR = 50% white clover:50% late perennial ryegrass; RC = red clover; WC = white clover.

[2]Value for clover treatments is given. SEM for the 4 grass treatments is slightly lower.

[3]G.C = EPR, FEST, TF, and LPR (grass) vs. RC and WC (clover); R.W = RC-LPR and RC vs. WC-LPR and WC; RC.L = linear effect of increasing RC proportion; RC.Q = quadratic effect of increasing RC proportion; WC.L = linear effect of increasing WC proportion; WC.Q = quadratic effect of increasing WC proportion.

**Table 5**. Effect of forage source on fecal output and composition, apparent total-tract digestibility of nutrients, and amount apparently digested in dairy cows

| | Treatment[1] | | | | | | | | | Contrast[3] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | EPR | FEST | TF | LPR | RC-LPR | WC-LPR | RC | WC | SEM[2] | G.C | R.W | RC.L | RC.Q | WC.L | WC.Q |
| No. of cows | 19 | 20 | 20 | 20 | 16 | 16 | 16 | 16 | | | | | | | |
| Fecal DM output, kg/d | 4.31$^{e}$ | 4.98$^{cd}$ | 4.67$^{de}$ | 4.54$^{de}$ | 5.54$^{b}$ | 5.48$^{b}$ | 6.14$^{a}$ | 5.28$^{bc}$ | 0.15 | <0.01 | <0.01 | <0.01 | 0.16 | <0.01 | <0.01 |
| Fecal composition | | | | | | | | | | | | | | | |
| DM, g/kg of fresh matter | 87.9$^{de}$ | 98.3$^{bc}$ | 84.9$^{e}$ | 93.0$^{cd}$ | 104$^{b}$ | 96.7$^{bc}$ | 115$^{a}$ | 103$^{b}$ | 2.81 | <0.01 | <0.01 | <0.01 | 0.91 | <0.01 | 0.64 |
| Ash, g/kg of DM | 153$^{e}$ | 141$^{f}$ | 141$^{f}$ | 131$^{g}$ | 166$^{d}$ | 176$^{c}$ | 207$^{b}$ | 218$^{a}$ | 2.44 | <0.01 | <0.01 | <0.01 | 0.28 | <0.01 | 0.53 |
| CP, g/kg of DM | 233$^{b}$ | 214$^{cd}$ | 202$^{e}$ | 194$^{f}$ | 211$^{d}$ | 222$^{c}$ | 216$^{cd}$ | 243$^{a}$ | 2.31 | <0.01 | <0.01 | <0.01 | 0.01 | <0.01 | 0.17 |
| NDF, g/kg of DM | 322$^{d}$ | 393$^{bc}$ | 411$^{b}$ | 452$^{a}$ | 377$^{c}$ | 315$^{d}$ | 341$^{d}$ | 237$^{e}$ | 7.62 | <0.01 | <0.01 | <0.01 | 0.02 | <0.01 | <0.01 |
| ADF, g/kg of DM | 145$^{e}$ | 188$^{c}$ | 194$^{bc}$ | 219$^{a}$ | 207$^{ab}$ | 163$^{d}$ | 217$^{a}$ | 142$^{e}$ | 4.12 | 0.02 | <0.01 | 0.55 | 0.02 | <0.01 | <0.01 |
| Feces score[4] | 2.2$^{cd}$ | 2.7$^{ab}$ | 2.6$^{bc}$ | 2.8$^{ab}$ | 2.8$^{ab}$ | 2.1$^{d}$ | 3.1$^{a}$ | 2.0$^{d}$ | 0.10 | 0.71 | <0.01 | 0.01 | 0.13 | <0.01 | 0.01 |
| Digestibility, % | | | | | | | | | | | | | | | |
| DM | 78.6$^{a}$ | 75.5$^{bc}$ | 75.1$^{bc}$ | 76.2$^{b}$ | 73.6$^{cd}$ | 74.6$^{bc}$ | 72.0$^{d}$ | 75.5$^{bc}$ | 0.57 | <0.01 | <0.01 | <0.01 | 0.36 | 0.24 | 0.04 |
| OM | 80.2$^{a}$ | 76.9$^{bc}$ | 76.7$^{bc}$ | 77.7$^{b}$ | 75.7$^{c}$ | 76.9$^{bc}$ | 75.1$^{c}$ | 78.4$^{ab}$ | 0.53 | <0.01 | <0.01 | <0.01 | 0.25 | 0.21 | 0.03 |
| CP | 70.9$^{bc}$ | 68.5$^{d}$ | 71.9$^{b}$ | 71.2$^{b}$ | 69.2$^{cd}$ | 72.7$^{b}$ | 71.6$^{b}$ | 77.8$^{a}$ | 0.53 | <0.01 | <0.01 | 0.58 | <0.01 | <0.01 | <0.01 |
| NDF | 78.1$^{a}$ | 71.5$^{b}$ | 72.6$^{b}$ | 70.8$^{b}$ | 68.2$^{b}$ | 71.7$^{b}$ | 60.7$^{c}$ | 70.7$^{b}$ | 1.16 | <0.01 | <0.01 | <0.01 | 0.05 | 0.95 | 0.42 |
| ADF | 81.1$^{a}$ | 74.6$^{b}$ | 74.2$^{b}$ | 73.7$^{bc}$ | 69.7$^{c}$ | 74.7$^{b}$ | 62.2$^{d}$ | 72.9$^{bc}$ | 1.09 | <0.01 | <0.01 | <0.01 | 0.13 | 0.55 | 0.23 |
| Amount digested, kg/d | | | | | | | | | | | | | | | |
| DM | 15.9$^{ab}$ | 15.3$^{bc}$ | 14.1$^{d}$ | 14.5$^{cd}$ | 15.3$^{bc}$ | 16.0$^{ab}$ | 15.7$^{ab}$ | 16.4$^{a}$ | 0.28 | <0.01 | <0.01 | <0.01 | 0.39 | <0.01 | 0.04 |
| OM | 14.9$^{ab}$ | 14.3$^{bc}$ | 13.2$^{d}$ | 13.7$^{cd}$ | 14.4$^{abc}$ | 15.0$^{ab}$ | 14.6$^{abc}$ | 15.2$^{a}$ | 0.26 | <0.01 | 0.01 | <0.01 | 0.41 | <0.01 | 0.03 |
| CP | 2.43$^{cd}$ | 2.30$^{de}$ | 2.40$^{d}$ | 2.17$^{e}$ | 2.61$^{c}$ | 3.25$^{b}$ | 3.35$^{b}$ | 4.63$^{a}$ | 0.06 | <0.01 | <0.01 | <0.01 | 0.01 | <0.01 | 0.01 |
| NDF | 4.99$^{a}$ | 4.95$^{a}$ | 5.07$^{a}$ | 4.96$^{a}$ | 4.48$^{b}$ | 4.33$^{b}$ | 3.16$^{c}$ | 3.01$^{c}$ | 0.11 | <0.01 | 0.11 | <0.01 | <0.01 | <0.01 | <0.01 |
| ADF | 2.69$^{a}$ | 2.78$^{a}$ | 2.61$^{a}$ | 2.79$^{a}$ | 2.64$^{a}$ | 2.62$^{a}$ | 2.15$^{b}$ | 2.03$^{b}$ | 0.06 | <0.01 | 0.19 | <0.01 | 0.01 | <0.01 | <0.01 |

$^{a–g}$Values within same line with different superscripts differ ($P < 0.05$).

[1]EPR = early perennial ryegrass; FEST = festulolium; TF = tall fescue; LPR = late perennial ryegrass; RC-LPR = 50% red clover:50% late perennial ryegrass; WC-LPR = 50% white clover:50% late perennial ryegrass; RC = red clover; WC = white clover.

[2]Value for clover treatments is given. SEM for the 4 grass treatments is slightly lower.

[3]G.C = EPR, FEST, TF, and LPR (grass) vs. RC and WC (clover); R.W = RC-LPR and RC vs. WC-LPR and WC; RC.L = linear effect of increasing RC proportion; RC.Q = quadratic effect of increasing RC proportion; WC.L = linear effect of increasing WC proportion; WC.Q = quadratic effect of increasing WC proportion.

[4]Determined on a 5-point (1–5; 1 is loose and 5 is firm) visual observation scale.

### Water Intake and Eating Behavior

Water intake through feed was dependent on TMR DM concentration, which also affected free water intake (Table 6). Total water intake varied from 101 to 110 L/d on the grass treatments, which was lower than total water intake on the clover treatments (114–128 L/d). White clover resulted in higher total water intake, increased duration of total drinking time and of each drinking bout, more drinking bouts, and reduced drinking rate compared with red clover. Increased clover proportion resulted in a linear increase in total water intake, drinking duration, and drinking bouts for both red clover and white clover. Water intake per drinking bout varied from 11.1 to 12.8 L, was larger for TF than for FEST and RC, and was not affected by clover proportion. The drinking bout duration for WC was longer than for all other treatments (2.81 vs. 1.91–2.28 min; $P < 0.05$), which also reduced drinking rate, as water intake per bout on WC did not differ from the other treatments.

Cows fed the WC diet spent 0.9 to 1.4 h less per day at the feeding through than cows on the other treatments ($P < 0.05$; Table 6). Feeding with LPR compared with EPR increased eating duration by 0.5 h/d but reduced the number of meals from 9.5 to 8.1/d. Eating rate was higher for EPR and FEST than for TF and LPR (80.5 and 81.1 vs. 70.3 and 68.2 g of DM/min), but meal size did not differ between grass treatments. Both red clover and white clover proportion decreased the total eating time and meal duration linearly, but linearly increased the number of meals and eating rate. Red clover proportion did not affect meal size, but white clover proportion resulted in a quadratic effect, as meal size for LPR and WC-LPR did not differ but WC reduced meal size by 0.54 kg of DM.

## DISCUSSION

### Silages

Both cuts of perennial ryegrass achieved exactly the planned DM concentration of 350 g/kg. The higher leaf-to-stem ratio for tall fescue compared with early perennial ryegrass probably speeded up the drying process, resulting in a higher DM concentration as opposed to festulolium. The achieved DM concentrations for the clover silages were 301 and 304 g/kg, as we assessed the risk for loosing leaf material to be too high if wilting continued to a higher DM concentration. All 6 silages were well preserved, as all reached a low pH, with lactate as the most dominating fermentation product without any detectable amount of butyrate (McDonald et al., 1991). Legumes have a higher buffer capacity than grasses (McDonald and Henderson, 1962), resulting in a higher amount of lactate needed to reach a stable pH, which also was seen in our red and white clover silages. Late perennial ryegrass silage had higher NDF, ADF, ADL, NDSF, and iNDF concentrations and a lower leaf-to-stem ratio, CP concentration, and OMD than early perennial ryegrass silage due to the increased maturity, which is consistent with other studies (Kuoppala et al., 2008; Alstrup et al., 2016). In the grass silages, the low ADL concentrations resulting in high iNDF-to-ADL ratios (6.2–9.8) were in contrast to earlier reported iNDF-to-ADL ratios for grass silages of 2.3 to 3.4 (Krämer et al., 2012). The difference might be due to different methodologies used to determine

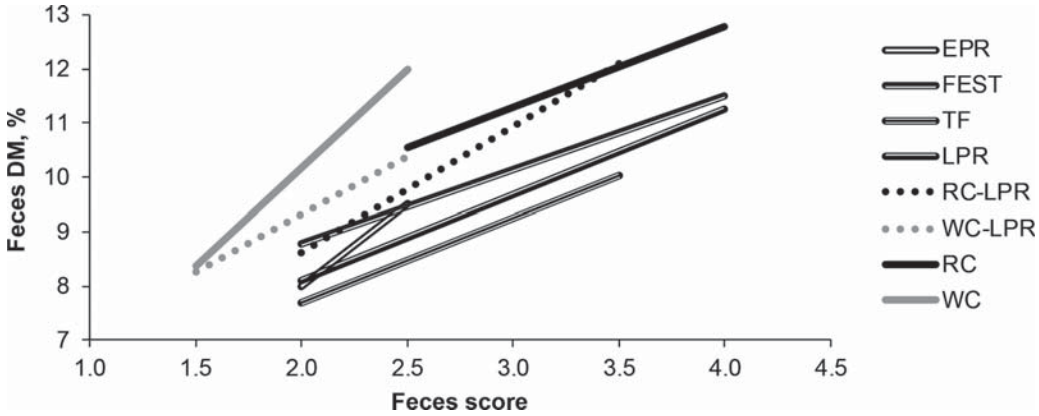


**Figure 1**. Fecal DM concentration as a function of feces score determined on a 5-point (1–5; 1 is loose and 5 is firm) visual observation scale for dairy cows fed a TMR based on different forage silages (EPR = early perennial ryegrass; FEST = festulolium; TF = tall fescue; LPR = late perennial ryegrass; RC-LPR = 50% red clover:50% late perennial ryegrass; WC-LPR = 50% white clover:50% late perennial ryegrass; RC = red clover; WC = white clover).

**Table 6**. Effect of forage source on water intake and drinking and eating behavior

| | Treatment[1] | | | | | | | | | Contrast[3] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | EPR | FEST | TF | LPR | RC-LPR | WC-LPR | RC | WC | SEM[2] | G.C | R.W | RC.L | RC.Q | WC.L | WC.Q |
| No. of cows | 19 | 20 | 20 | 20 | 16 | 16 | 16 | 16 | | | | | | | |
| Water intake | | | | | | | | | | | | | | | |
| Feed water, L/d | 26.4$^{c}$ | 34.3$^{a}$ | 18.9$^{d}$ | 25.8$^{c}$ | 31.2$^{b}$ | 31.8$^{b}$ | 35.4$^{a}$ | 34.6$^{a}$ | 0.53 | <0.01 | 0.90 | <0.01 | 0.30 | <0.01 | <0.01 |
| Free water, (L/d)$^{-1}$ | 0.013$^{de}$ | 0.013$^{de}$ | 0.011$^{ab}$ | 0.013$^{e}$ | 0.013$^{de}$ | 0.012$^{bc}$ | 0.013$^{cd}$ | 0.011$^{a}$ | 0.0003 | <0.01 | <0.01 | <0.01 | 0.75 | <0.01 | 0.21 |
| Free water,[4] L/d | 78.2 | 76.0 | 90.1 | 74.9 | 77.7 | 85.4 | 79.7 | 93.6 | | | | | | | |
| Total water, (L/d)$^{-1}$ | 0.010$^{de}$ | 0.009$^{c}$ | 0.009$^{cd}$ | 0.010$^{e}$ | 0.009$^{cd}$ | 0.009$^{b}$ | 0.009$^{bc}$ | 0.008$^{a}$ | 0.0002 | <0.01 | <0.01 | <0.01 | 0.29 | <0.01 | 0.04 |
| Total water,[4] L/d | 105 | 110 | 108 | 101 | 109 | 116 | 114 | 128 | | | | | | | |
| Drinking behavior | | | | | | | | | | | | | | | |
| Duration, $\log_{10}$(min/d) | 1.17$^{bc}$ | 1.13$^{cd}$ | 1.20$^{b}$ | 1.10$^{d}$ | 1.10$^{d}$ | 1.20$^{b}$ | 1.18$^{bc}$ | 1.36$^{a}$ | 0.027 | <0.01 | <0.01 | <0.01 | 0.06 | <0.01 | 0.17 |
| Duration,[4] min/d | 14.9 | 13.4 | 15.9 | 12.6 | 12.6 | 15.9 | 15.0 | 23.1 | | | | | | | |
| Bouts, number/d | 6.8$^{b}$ | 6.8$^{b}$ | 7.1$^{b}$ | 6.7$^{b}$ | 7.0$^{b}$ | 7.3$^{b}$ | 7.3$^{b}$ | 8.5$^{a}$ | 0.45 | <0.01 | <0.01 | 0.04 | 0.92 | <0.01 | 0.22 |
| Bout duration, $\log_{10}$(min) | 0.35$^{bc}$ | 0.30$^{bcd}$ | 0.37$^{b}$ | 0.30$^{cd}$ | 0.28$^{d}$ | 0.36$^{bc}$ | 0.33$^{bcd}$ | 0.45$^{a}$ | 0.025 | <0.01 | <0.01 | 0.13 | 0.11 | <0.01 | 0.48 |
| Bout duration,[4] min | 2.26 | 2.01 | 2.32 | 1.98 | 1.91 | 2.28 | 2.15 | 2.81 | | | | | | | |
| Intake per bout, (L)$^{-1}$ | 0.087$^{ab}$ | 0.089$^{b}$ | 0.078$^{a}$ | 0.086$^{ab}$ | 0.087$^{ab}$ | 0.084$^{ab}$ | 0.090$^{b}$ | 0.086$^{ab}$ | 0.0049 | 0.17 | 0.21 | 0.29 | 0.57 | 0.84 | 0.60 |
| Intake per bout,[4] L | 11.5 | 11.3 | 12.8 | 11.6 | 11.6 | 11.8 | 11.1 | 11.7 | | | | | | | |
| Drinking rate, $\log_{10}$(L/min) | 0.72$^{b}$ | 0.76$^{ab}$ | 0.76$^{ab}$ | 0.78$^{ab}$ | 0.80$^{a}$ | 0.73$^{ab}$ | 0.73$^{ab}$ | 0.62$^{c}$ | 0.027 | <0.01 | <0.01 | 0.05 | 0.05 | <0.01 | 0.13 |
| Drinking rate,[4] L/min | 5.3 | 5.8 | 5.7 | 6.0 | 6.3 | 5.4 | 5.4 | 4.2 | | | | | | | |
| Eating behavior | | | | | | | | | | | | | | | |
| Duration, h/d | 4.10$^{b}$ | 4.12$^{b}$ | 4.37$^{ab}$ | 4.60$^{a}$ | 4.46$^{ab}$ | 4.17$^{b}$ | 4.15$^{b}$ | 3.19$^{c}$ | 0.13 | <0.01 | <0.01 | <0.01 | 0.47 | <0.01 | 0.02 |
| Meals, number/d | 9.5$^{b}$ | 9.2$^{bc}$ | 8.4$^{cd}$ | 8.1$^{d}$ | 9.4$^{bc}$ | 9.4$^{bc}$ | 10.0$^{b}$ | 11.6$^{a}$ | 0.30 | <0.01 | 0.01 | <0.01 | 0.23 | <0.01 | 0.14 |
| Meal duration, min | 27.1$^{c}$ | 28.3$^{bc}$ | 32.5$^{ab}$ | 35.1$^{a}$ | 29.6$^{bc}$ | 28.7$^{bc}$ | 26.2$^{c}$ | 17.1$^{d}$ | 1.41 | <0.01 | <0.01 | <0.01 | 0.44 | <0.01 | 0.06 |
| Meal size, kg of DM | 2.25$^{ab}$ | 2.37$^{a}$ | 2.35$^{a}$ | 2.46$^{a}$ | 2.33$^{a}$ | 2.48$^{a}$ | 2.30$^{a}$ | 1.93$^{b}$ | 0.09 | <0.01 | 0.16 | 0.14 | 0.60 | <0.01 | <0.01 |
| Eating rate, (g of DM/min)$^{-1}$ | 0.012$^{b}$ | 0.012$^{b}$ | 0.014$^{cd}$ | 0.015$^{d}$ | 0.013$^{bc}$ | 0.012$^{b}$ | 0.012$^{b}$ | 0.009$^{a}$ | 0.0004 | <0.01 | <0.01 | <0.01 | 0.60 | <0.01 | 0.97 |
| Eating rate,[4] g of DM/min | 80.5 | 81.1 | 70.3 | 68.2 | 76.4 | 83.2 | 84.2 | 106.4 | | | | | | | |

[a–e]Values within same line with different superscripts differ ($P < 0.05$).

[1]EPR = early perennial ryegrass; FEST = festulolium; TF = tall fescue; LPR = late perennial ryegrass; RC-LPR = 50% red clover:50% late perennial ryegrass; WC-LPR = 50% white clover:50% late perennial ryegrass; RC = red clover; WC = white clover.

[2]Value for clover treatments is given. SEM for the 4 grass treatments is slightly lower.

[3]G.C = EPR, FEST, TF, and LPR (grass) vs. RC and WC (clover); R.W = RC-LPR and RC vs. WC-LPR and WC; RC.L = linear effect of increasing RC proportion; RC.Q = quadratic effect of increasing RC proportion; WC.L = linear effect of increasing WC proportion; WC.Q = quadratic effect of increasing WC proportion.

[4]Back-transformed LSM from the above transformed data.

ADL, as the Ankom procedure was used in our study. Postponing harvest of perennial ryegrass 13 d did not reduce OMD to the level of tall fescue silage, which was the silage with the highest NDF concentration and lowest OMD. Based on the leaf-to-stem ratio, which was very high in tall fescue, this was not expected, as cell wall concentrations normally are lower in leaves than in stems (Wilson, 1994; Buxton and Redfearn, 1997). Leaves of tall fescue have a higher number of sclerenchyma bundles with a higher average area compared with perennial ryegrass, and each single fiber is longer and broader (King et al., 2014). This can probably explain the lower OMD of tall fescue silage than of perennial ryegrass silage. The higher concentration of NDSF demonstrated that legumes contain more pectin substances than grasses (Wilson, 1994).

Red clover silage had a lower concentration of soluble N and ammonia N as proportion of total N compared with the other silages, which may be related to the presence of polyphenol oxidases in red clover (Lee, 2014), resulting in a larger insoluble, but rumen-degradable, protein fraction. Broderick and Albrecht (1997) demonstrated a lower rumen degradation rate of protein in red clover than in white clover, but in our study the fractional rate of protein degradation was similar for white clover and red clover, which was substantially higher than for the grass species. However, the ruminal protein degradation rate in red clover can vary from 7.5 to 35.5%/h depending on growth stage and conservation method (Aufrère et al., 2002). The fractional rate of rumen NDF degradation for red clover silage was comparable to values reported by Hoffman et al. (1997), similar to the degradation rate of NDF for white clover silage and higher than that of the grass silages. Generally, legumes have a higher rate of rumen NDF degradation than grasses, but it is highly dependent on maturity stage (Hoffman et al., 1993).

### Feed Intake

As shown in previous studies (Steinshamn, 2010; Johansen et al., 2017), our study confirmed that DMI is higher in cows fed legumes compared with cows fed grasses. The quadratic effect of white clover proportion on DMI and the lower BW in the WC treatment compared with the other treatments indicated that rumen fill probably did not restrict DMI on the WC treatment, but that DMI probably instead was regulated physiologically (Mertens, 1994). Dewhurst et al. (2003a) and Bertilsson and Murphy (2003) weighed rumen content and found that cows fed white clover had 18 to 20 and 16.5 kg less material in the rumen, respectively, compared with cows fed grass, red clover, or grass-clover mixes. Thereby, a lower rumen fill, and not loss of body mass, was most probably causing the lower BW on the WC treatment in our study. A notable and immediate drop in BW when cows shifted to WC and RC (data not shown) also supported this. After the drop, both groups increased BW during the first days in the period because of increasing DMI. However, cows on the RC treatment reached the same BW as cows on grass treatments in the end of the period indicating rumen fill to be the same, whereas cows on the WC treatment did not reach the same BW as cows on the other treatments. The DMI was stable for both RC and WC in the last week of the period; therefore, compared with the other treatments, DMI on the WC treatment was not as high as expected based on silage OMD, probably because of a different regulation of DMI. The same was applicable for milk production, which reflected DMI; however, cows on the WC treatment produced the expected amount of milk based on the amount of OM digested, which is discussed further herein.

In the grass treatments, TF and LPR resulted in a lower DMI compared with EPR and FEST, which may be related to silage OMD. Both FEST and EPR resulted in a similar DMI despite the fact that early perennial ryegrass silage had a higher OMD and a lower concentration of NDF than festulolium silage; therefore, EPR was expected to result in a higher DMI than FEST. To some extent, this might indicate metabolic intake regulation for EPR, such as for WC.

### Milk Yield and Milk Composition

The higher milk and ECM yield for cows fed clover compared with cows fed grass, as well as the higher milk and ECM yield for cows fed white clover compared with cows fed red clover, corresponded with earlier findings (Steinshamn, 2010; Johansen et al., 2017). We did not detect differences in milk composition between cows fed red clover and white clover, but increasing the clover proportion resulted in a linear decrease in fat and protein concentrations and a linear increase in lactose concentration. Moorby et al. (2009) observed a linear decrease in fat and protein concentrations without any differences in lactose concentration, whereas Halmemies-Beauchet-Filleau et al. (2014) observed a linear increase in lactose concentration but no effect on fat concentration when increasing the red clover proportion in the diet. Vanhatalo et al. (2009) reported the same effect on milk fat, protein, and lactose concentrations as observed in our study when increasing the red clover proportion. Lactose is the main osmotic regulator in milk, and thereby the most important factor determining milk yield (Linn, 1988). In our study, lactose production per day increased significantly when increasing the clover proportion, resulting in a higher

milk yield. The higher milk production can probably explain the increased lactose concentration, because of a higher intramammary pressure, which increases the lactose concentration (Lollivier et al., 2002). However, Halmemies-Beauchet-Filleau et al. (2014) did not detect any differences in milk yield but reported lactose concentrations of 48.2 and 47.3 g/kg for cows fed red clover and grass silage, respectively, which is very similar to the lactose concentrations observed in our study.

The increase in daily milk protein production when increasing the clover proportion was probably caused by an increase in $NE_L$ intake (Linn, 1988). However, lactose production increased even more; thus, the observed changes in protein concentration in our study, when increasing clover proportion, most probably were caused by a dilution of the protein. However, a meta-analysis (Johansen et al., 2017) reported a lower milk protein concentration when cows were fed red clover compared with grasses and white clover, which could be caused by complexing of polyphenol oxidases with plant proteins, protecting proteins from degradation in the rumen and reducing bioavailability of sulfur-containing AA (Lee, 2014). However, our in situ-determined rumen protein degradation rate (Table 3) did not differ between white clover and red clover; thus, protein complexes formed by polyphenol oxidases were presumably not responsible for the reduced milk protein concentration when feeding red clover in our study, as similar milk protein concentrations were observed for red clover and white clover.

The decrease in milk fat concentration when increasing clover proportion may be due to changed molar VFA proportions in the rumen when feeding clover compared with grass, as the milk fat concentration is positively related to acetic and butyric acids proportion and negatively related to the propionic acid proportion (Linn, 1988). Fiber degradation stimulates the production of acetic and butyric acid and, according to Mertens (1985), an NDF concentration of 280 g/kg and an ADF concentration of 180 g/kg in the diet are needed as a minimum to maximize milk production and fat concentration. In our study, fiber concentrations in the RC and WC diets were below these limits, the RC-LPR and WC-LPR diets were close to the limits, and the LPR diet was above the limit (Table 1). This probably can explain the linear decrease in milk fat concentration when the clover proportion is increased. Even though not significant, the cows produced numerically more fat per day when fed RC-LPR compared with RC and LPR and when fed WC-LPR compared with WC and LPR. Vanhatalo et al. (2009) observed a decrease in milk fat concentration and a reduced rumen molar proportion of butyric acid when increasing the red clover proportion. Likewise, Halmemies-Beauchet-Filleau et al. (2013) reported a reduced ratio of rumen acetate and butyrate to propionate when increasing the red clover proportion. The reduced milk fat concentration when increasing the clover proportion could also be caused by an increased concentration of milk MUFA and PUFA when cows were fed clover compared with grass (Dewhurst et al., 2003b; Vanhatalo et al., 2007), as these can have an inhibitory effect on the de novo fatty acid synthesis in the mammary gland (Bauman and Griinari, 2003). An increase in milk MUFA and PUFA when feeding clover compared with grass is probably caused by a lower rumen biohydrogenation of polyunsaturated C18 fatty acids (Lejonklev et al., 2013).

The increase in milk fat concentration and decrease in milk protein concentration when the harvest of perennial ryegrass was postponed was in contrast to Vanhatalo et al. (2009), who did not observe any differences in milk composition when grass and red clover of different maturity stages were fed to dairy cows. However, the responses were in agreement with the aforementioned increases in milk protein production due to increased $NE_L$ intake and increased milk fat concentration due to higher NDF and ADF concentrations in the diet. Nevertheless, a decrease in milk yield counterbalanced the increase in milk fat concentration, whereby daily milk fat production was similar for EPR and LPR. For the grass treatments, FEST resulted in the highest milk fat production per day, which cannot be explained by the NDF and ADF concentrations in the diet.

### *Relation Between OMD and Milk Production*

The early perennial ryegrass silage had the highest OMD of the grass silages, but the EPR treatment did not result in the highest milk production. A high passage rate could cause an inefficient digestion of the nutrients, but the total-tract digestibility of DM, OM, NDF, and ADF were higher for EPR than the other grass treatments; therefore, a higher passage rate was probably not the cause of the lower than expected milk production. Figure 2a relates ECM to the amount of OM digested in the gastrointestinal tract. The correlation between ECM and the amount of OM digested was generally high ($R^2 = 0.78$) across the 8 treatments, but without the EPR treatment coefficient of determination increased to 0.98. Related to the amount of OM digested, the cows produced 2.6 kg of ECM less than expected on the EPR treatment compared with the production on the other treatments. An explanation could be that the energy concentration in the digested OM is lower on the EPR treatment than on the other treatments, but nothing in the chemical composition of the silages (Table 2) can explain such a difference and the calculated $NE_L$ intake (Table 4) was higher

for EPR than for the other grass treatments. Similarly, in a study by Randby et al. (2012), cows fed a very early harvested, highly digestible grass-clover silage did not produce the milk yield expected from the chemical composition compared with grass-clover silages harvested at later stages of crop maturity with lower OM digestibility. In our study, similar amounts of OM were digested per day on EPR, WC-LPR, and WC; thus, it cannot be the high amount of digested OM that explains the lower utilization of digested OM to milk production on EPR. Cows fed WC digested twice as much CP, but digested 40% less NDF compared with cows fed grass (Table 5); however, cows fed WC produced the expected amount of milk based on the amount of total OM digested compared with the other treatments. Secretion of surplus nitrogen via urea is an energy-demanding process, and therefore lower energy efficiency could have been expected for cows fed WC. However, Alstrup et al. (2016) reported no negative effects on milk production in dairy cows fed diets with 23.1% CP of DM, even though large amounts of urea were secreted in milk. The efficiency of protein utilization was similar between grass treatments (31, 32, 29, and 33% for EPR, FEST, TF, and LPR, respectively) and was lower in the clover treatments than in the grass treatments, but across treatments the efficiency of protein utilization was highly correlated with the TMR CP level. Therefore, the higher proportion of soluble N in early perennial ryegrass silage compared with the other silages cannot explain the lower than expected milk production. Moreover, BW gain cannot explain the lower than expected milk production in cows fed EPR, neither in our study nor in the study by Randby et al. (2012).

In Figure 2b, ECM is related to silage OMD, and the treatments FEST, TF, and LPR represent a straight line indicating that silage OMD up to at least 80.6% can explain the variation in ECM between different grass species; 1 unit increase in silage OMD increased ECM with 0.6 kg/d. Including 50% clover in the diet increased ECM with 2.3 kg/d compared with the pure grass diets when silage OMD was comparable, independently of clover species, and change in silage OMD resulted in the same response in ECM as for the grass treatments. We expected that feeding 100% clover would increase ECM further and give the same response to changes in silage OMD if rumen fill regulates the intake. However, this was probably not the case for WC, as previously discussed; thus, our data cannot substantiate this. If rumen fill regulates intake, our data indicated that the response in ECM was higher when increasing clover proportion from 0 to 50% than from 50 to 100%, as RC-LPR increased ECM with 1.9 kg/d compared with LPR whereas RC only increased ECM with 0.5 kg/d compared with RC-LPR. Figure 2b indicates an optimum for silage OMD in relation to ECM production, and the relationship between ECM and silage OMD can probably be quadratic with separate lines for grass- and clover-containing diets, respectively. Cows on EPR and WC, which were the treatments including the silages with the highest OMD, responded differently compared with cows on the other treatments, as already discussed. Although the optimum could not be determined exactly, our data indicates that the optimum for silage OMD is within the range 79 to 82%.

Figure 2c relates the apparent total-tract OM digestibility to silage OMD. For the treatments with a silage OMD of 76 to 77% we noted high agreement to the apparent total-tract digestibility, whereas treatments with a higher silage OMD were below the identity line and vice versa. Values reported by Kuoppala et al. (2009) indicate similar tendencies when comparing silage OM digestibility obtained in vivo in sheep fed at maintenance and total-tract OM digestibility in producing dairy cows fed silage ad libitum and concentrate. Compared with the other grass treatments, total-tract OM digestibility for FEST differed more from silage OMD, and the deviation was comparable to the deviations obtained for the 2 treatments containing white clover. When comparing ECM to apparent total-tract OM digestibility (Figure 2d), FEST is located closer to the 4 clover treatments than to the other grass treatments. The festulolium silage had an iNDF concentration comparable to the tall fescue silage, but the fractional rate of NDF degradation was 5.12%/h for festulolium silage compared with 4.06%/h for tall fescue silage (Table 3). Therefore, the shape of the NDF degradation curve for festulolium silage differed from the other 3 grass silages, which were more or less similar in shape. Instead, the shape of the NDF degradation curve for festulolium silage was more toward the shape of the 2 clover silages, which were also similar in shape. Based on the total-tract NDF digestibility and the NDF degradation curves for the silages, the fractional rate of passage for NDF could be calculated as 0.83, 1.24, 0.96, 0.95, 1.61, and 1.09%/h in EPR, FEST, TF, LPR, RC, and WC, respectively. This indicates that the fiber in festulolium behaves more like fiber in legumes when fed to dairy cows, but this is not reflected in silage OMD. The explanation for this needs to be studied further.

### *CP Digestibility and Feces*

The total-tract CP digestibility reflected the disappearance of CP from mobile bags, as FEST resulted in a lower total-tract CP digestibility than EPR and TF and white clover resulted in a higher total-tract CP digestibility than red clover. Generally, a higher

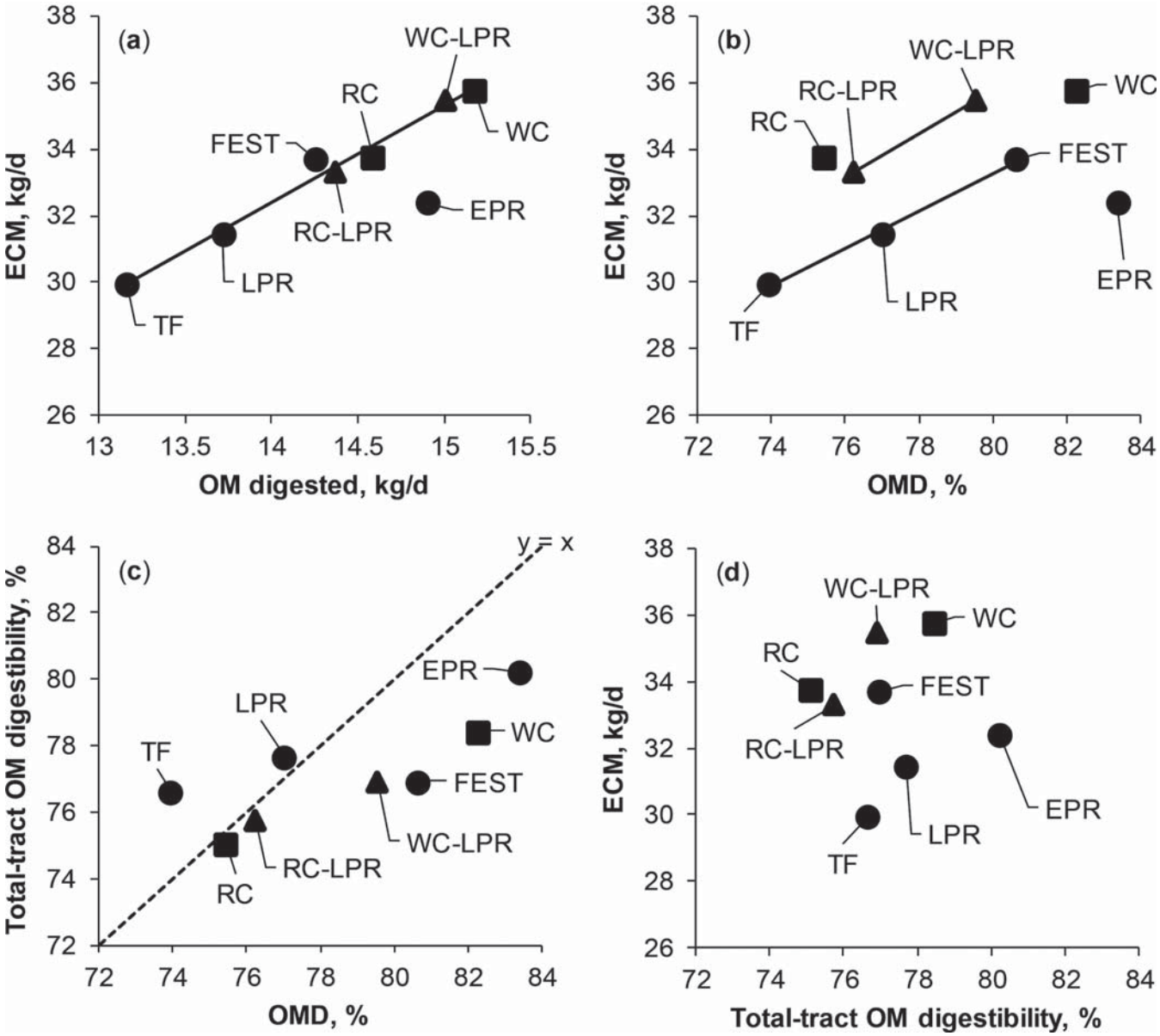


**Figure 2**. Relationship between (a) ECM and amount of OM digested in the gastrointestinal tract with the regression line ($R^2 = 0.98$) across treatments without EPR; (b) ECM and silage in vivo OM digestibility (OMD) calculated as 4.10 + 0.959 × in vitro OM digestibility (Møller et al., 1989); (c) total-tract OM digestibility and OMD; and (d) ECM and total-tract OM digestibility for dairy cows fed TMR based on different forage silages (EPR = early perennial ryegrass; FEST = festulolium; TF = tall fescue; LPR = late perennial ryegrass; RC-LPR = 50% red clover:50% late perennial ryegrass; WC-LPR = 50% white clover:50% late perennial ryegrass; RC = red clover; WC = white clover).

feed CP concentration increased CP digestibility, both when comparing silage CP concentration with disappearance of CP from mobile bags and when comparing TMR CP concentration with total-tract CP digestibility. However, total-tract CP digestibility was lower for FEST and RC-LPR than for EPR, TF, and LPR even though the TMR CP concentration was similar. The lower total-tract CP digestibility for red clover compared with grass and white clover is in accordance with Dewhurst et al. (2003b).

Despite similar fecal DM concentrations, feces scores were lower for WC-LPR and WC compared with the other treatments. The missing correlation between fecal DM concentration and fecal consistency has also been observed by Ireland-Perry and Stallings (1993) in dairy cows fed diets differing in ADF concentration. Mgbea-

huruike et al. (2016) reported a positive correlation between diet NDF concentration and fecal consistency; however, differences in dietary or fecal concentrations of major nutrients could not explain the differences in feces scores observed in our study. Different plant fibers have different water binding capacity (Chen et al., 1984), indicating that the fiber fraction in white clover silage differs in water binding capacity compared with the fiber fraction in the other silages resulting in the more liquid feces.

### Drinking and Eating Behavior

Daily free water intakes in our study correspond well to previously published data for lactating cows [e.g., 89.5 L/d (Dado and Allen, 1994) and 83.6 L/d (Cardot et al., 2008)]. Water intake is affected by many factors, including DMI, milk yield, feed DM concentration, weather conditions (temperature and rainfall), and CP and potassium intakes (Cardot et al., 2008; Kume et al., 2010). The higher water intake when feeding clover compared with grass in our study was probably a combination of higher DMI, higher milk yield, and higher CP intake. White clover and red clover did not differ in DMI; thus, presumably it was the difference in milk yield and CP intake, which caused the difference in water intake between red clover and white clover.

In our study, the average number of drinking bouts of 7.2 corresponds well to 7.3 drinking bouts/d as reported by Cardot et al. (2008). The drinking behavior for cows on WC was notably different from the other treatments, which did not differ substantially. The water intake per drinking bout in WC was similar to the other treatments, resulting in an increased number of drinking bouts per day to increase free water intake. However, the duration of each drinking bout was longer, making the drinking rate slower for cows offered WC, resulting in total drinking duration to be 60% higher for cows fed WC compared with the other treatments. The drinking rate is affected by water flow rate to the water-bowl and social rank (Andersson et al., 1984), but these factors cannot explain the difference between treatments observed in our study, as the cows drank from a drinking trough with a free water surface that was filled up between visits and cows stayed in the same group throughout the experiment. Whether rumen fill and rumen NDF concentration affect drinking rate is unknown, but these are factors that we expect to have had an influence on the drinking behavior in the WC treatment. The longer drinking duration for cows fed WC could also be an indirect effect of the lesser time spent eating.

Eating rate is negatively correlated with NDF and ADF concentrations in the diet (McLeod and Smith, 1989), which can explain the higher eating rate for white clover than for red clover and the linear increase in eating rate when increasing the clover proportion. The increased eating rate reduced total eating duration per day, even though total DMI was higher when increasing the clover proportion and when comparing EPR and LPR. Clover proportion or grass species did not affect meal size; thus, an increased eating rate reduced meal duration. Furthermore, the unchanged meal size increased meals per day when total DMI was higher, which is in contrast to findings by Beauchemin et al. (2002) and Dewhurst et al. (2003b), where similar numbers of larger meals were associated with increased intake. However, cows fed the WC diet on average consumed 0.43 kg of DM less per meal than cows fed the other diets, resulting in more meals in WC than WC-LPR, even though DMI did not differ. When rumen fill regulates intake, distension of receptors in the rumen wall stimulates the cow to end a meal (Allen, 2000). On the contrary, when feeding high levels of concentrate or high-quality silages, the high production of VFA over a short period stimulates ruminal epithelial receptors resulting in meal cessation, which reduces meal duration and meal size (Allen, 2000). As white clover silage presumably has a rapid rate of ruminal fermentation, the stimulation of ruminal epithelial receptors can probably explain the smaller meal size and the shorter meal duration for cows fed WC.

### Applications

Our findings indicated that production responses within both grass and clover species could be predicted based on in vitro OMD analyses of the silage. This is a useful tool for farmers regarding optimization of forage and milk production. Normally, grass and clover are cultivated in mixed swards, but the clover proportion can vary widely due to management and seasonal differences (Søegaard, 2009; Eriksen et al., 2014). Therefore, when evaluating the milk production potential of a grass-clover mixture based on in vitro OMD analyses of the silage, it is important to know the clover proportion, as clover inclusion up to at least 50% will increase milk production compared with pure grass at the same silage OMD level. To optimize profitability, the farmers should consider not only harvest yield and production costs, but also digestibility and inclusion rate of clover, and hereupon select the species most suitable for local conditions.

## CONCLUSIONS

Differences in ECM in cows fed silages of different grass species could be explained by differences in silage

OMD; likewise, at comparable silage OMD, inclusion of clover in the diets increased ECM. Cows fed grass silage with a high OMD (83.4%) did not produce the expected amount of ECM based on the amount of OM actually digested in the gastrointestinal tract. The results indicated that feed intake when feeding pure white clover was regulated physiologically instead of physically and, simultaneously, the eating and drinking behavior differed markedly from what of cows fed the other silages.

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## 5.3 Paper III – Metabolisable protein supply to lactating dairy cows increased with increasing dry matter concentration in grass-clover silage

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# Metabolisable protein supply to lactating dairy cows increased with increasing dry matter concentration in grass-clover silage

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ABSTRACT

The aim of this experiment was to study the effect of increased dry matter (DM) concentration in grass-clover silage, obtained by extending the pre-wilting period before ensiling, on the amount of metabolisable protein (MP) supplied to lactating dairy cows. Spring growth and first regrowth of grass-clover swards grown by two Danish organic farmers were cut and pre-wilted to a planned DM concentration of 350 and 700 g/kg, respectively, giving in total eight silages with DM concentrations ranging from 283 to 725 g/kg. Four Holstein dairy cows in late lactation with fistulae in rumen, duodenum and ileum were included in a crossover design, with five periods of 21 d. The cows were fed *ad libitum* with the experimental silages without any concentrate, but with daily supply of minerals and vitamins. Feed intake was registered daily and in the last week of each period 12 subsamples of duodenal and ileal chyme and faeces, respectively, were collected over 94 h to cover the diurnal variation, pooled, and subsequently analysed. Rumen fluid was collected in same sampling procedure. To estimate the duodenal flow of microbial protein, microbes were isolated from the rumen and analysed for amino acids (AA) and purines. Methane ($CH_4$) production was measured the last two days in each period in open-circuit respiration chambers. Results were analysed using a linear random regression model with DM concentration as fixed effect, cow and cut number x farmer as random intercepts and with a cut number x farmer random slope. The amount of AA digested in the small intestine increased ($P = 0.024$) by 5.59 g/kg DM intake with each increase in silage DM concentration of 100 g/kg. The increased digestion of AA in the small intestine was caused by a higher small intestinal digestibility of AA and a tendency towards a higher duodenal flow of AA. The higher duodenal flow of AA derived from a lower rumen degradation of feed protein and a tendency towards a higher microbial synthesis in the rumen. Fibre digestibility and $CH_4$ production were not affected by silage DM concentration. In conclusion, MP concentration in grass-clover silage can be improved by pre-wilting to a higher DM concentration before ensiling.

## 1. Introduction

A major factor affecting milk production in dairy cows is the amount of amino acids (AA) absorbed in the small intestine, defined as metabolisable protein (MP). Metabolisable protein depends on the amount of rumen undegraded feed protein (RUP),

*Abbreviations:* AA, amino acids; AA-N, amino acid nitrogen; ADL, acid detergent lignin; aNDFom, neutral detergent fibre assayed with heat stable amylase and expressed exclusive of residual ash; CP, crude protein; $CH_4$, methane; DM, dry matter; DMI, dry matter intake; ECM, energy corrected milk; iNDF, indigestible neutral detergent fibre; IVOMD, *in vitro* organic matter digestibility; MP, metabolisable protein; $NH_3$-N, ammonia nitrogen; NPN, non-protein nitrogen; OM, organic matter; OMD, *in vivo* organic matter digestibility; RUP, rumen undegraded feed protein; SCFA, short chain fatty acids; SE, standard error

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the amount of microbial true protein synthesised in the rumen and the amount of endogenous protein reaching the small intestine, and the digestibility of true protein in the small intestine (Allen, 1996). Organic feed rations for dairy cows in Denmark consist primarily of grass-clover silage and grain, making it difficult to fulfil the requirement for MP in high producing dairy cows, as the protein in grass-clover silage is degraded largely in the rumen (Tamminga et al., 1991) and a dearth of soluble carbohydrates as energy substrate reduces microbial efficiency (Fijalkowska et al., 2015). The protein value of grass-clover silage is affected by not only the total crude protein (CP) concentration in the crop, but also by the distribution between true protein nitrogen (N) and non-protein N (NPN). After mowing of the crop and in the initial ensiling process, some proteins are hydrolysed to peptides and free AA due to the activity of plant proteases and during ensiling ammonia is formed from breakdown of AA due to microbial fermentation (Kemble, 1956). The extent of proteolysis and the amount of ammonia produced are dependent of several factors such as forage species, cut number and wilting time (Papadopoulos and McKersie, 1983). Increased dry matter (DM) concentration of the crop reduces proteolysis during harvest and wilting (Slottner and Bertilsson, 2006) and inhibits microbial activity during the subsequent ensiling process due to increased osmotic pressure (McDonald et al., 1991). Therefore, DM concentration is negatively correlated to the amount of soluble NPN and is together with CP concentration the most important factor for predicting the amount of soluble NPN in forage silages (Muck et al., 1996). The amount of RUP measured *in situ* increases with increased DM concentration of grass silage due to the reduced amount of soluble NPN (van Vuuren et al., 1990; Tamminga et al., 1991; Verbic et al., 1999; Aufrere et al., 2003; Edmunds et al., 2014). Silages wilted to higher DM concentrations have a higher concentration of water soluble carbohydrates and a lower concentration of fermentation acids due to the restricted microbial fermentation (Gordon et al., 2000; Purwin et al., 2009; van Ranst et al., 2009), by which more fermentable matter is available for microbial fermentation in the rumen, increasing the microbial protein flow out of the rumen (Verbic et al., 1999).

In Denmark, normal practice is to pre-wilt grass-clover to a DM concentration of 300–350 g/kg prior to ensiling, as it is assumed most optimal in relation to minimising DM losses and to optimising ensilability. Increasing the DM concentration by extended pre-wilting will increase the risk of field DM losses but will presumably also increase the protein value of the silage. The protein metabolism has been studied in dairy cows fed either direct cut or wilted grass silages (Narasimhalu et al., 1989; Teller et al., 1992) and in dairy cows fed alfalfa silages wilted to different DM concentrations (Merchen and Satter, 1983). However, the protein metabolism, when feeding wilted grass silages with different DM concentrations, has been studied only in sheep (Verbic et al., 1999; Aufrere et al., 2003; Nguyen et al., 2005). Therefore, the main objective of this study was to investigate how pre-wilting of grass-clover to higher DM concentration before ensiling affects the *in vivo* nitrogen metabolism in the rumen and the amount of MP supplied to lactating dairy cows.

## 2. Materials and methods

The current experiment complied with the guidelines of Danish Ministry of Justice Law No. 726 (September 9, 1993) concerning animal experimentation and care of experimental animals.

### 2.1. Forages

The experimental grass-clover silages were produced during the growing season of 2013 by two organic farmers at their respective locations in Denmark; Varde (55°43′N 8°31′E; farm 1) and Skjern (55°59′N 8°35′E; farm 2). The grass-clover swards consisted of perennial ryegrass (*Lolium prenne*), hybrid ryegrass (*Lolium x boucheanum*), white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) at both farms and further of timothy (*Phlenum pratense*) and chicory (*Cichorium intybus*) at farm 1. Two cuts, the spring growth and the first regrowth, were produced at each farm to increase variation in chemical composition between cuts, making conclusion on the effect of DM concentration more universal. For each cut, the DM concentration after wilting was planned to 350 g/kg for the half and 700 g/kg for the remaining, giving in total eight silages. At farm 1, the spring growth was mown May 30 with a disc mower conditioner without crimper and wilted for 25 h and 120 h, respectively, and the first regrowth was mown July 5 with a disc mower conditioner with crimper and wilted for 69 h and 77 h, respectively. At farm 2, the spring growth was mown June 3 and wilted for 27 h and 100 h, respectively, and the first regrowth was mown July 8 and wilted for 24 h and 78 h, respectively. Both cuts at farm 2 were mown with a disc mower conditioner with crimper. At both farms, the wilted herbages were baled using a round baler and wrapped with plastic using same procedure and ensiled without additives. Six months later, the bales were transported to Research Centre Foulum, Tjele, Aarhus University, and re-wrapped with plastic immediately to cover damages arisen during transportation. Before the feeding experiment, two to four bales of the same silage were opened and mixed in a Cormall horizontal mixer for 30 min, and subsequently each silage was vacuum packed in approximately 200 polyethylene bags with 7–15 kg in each to ensure uniformity and quality of the silages during the feeding experiment. One bag of each silage was randomly selected for chemical analyses.

### 2.2. Animals and feeding

Four Danish Holstein cows, two first-parity and two second-parity, were used for the experiment. The cows were 216 ± 23 (mean ± SD) days from calving and weighed 551 ± 33 kg at the beginning of the experiment. Each cow was fitted with a cannula in the rumen (#1C, Bar Diamond Inc., Parma, ID), an open T-type cannula in duodenum placed 60 cm caudal to pylorus, and an open T-type cannula in ileum placed 20 cm cranial to the cecum. The cows were housed on rubber mats with sawdust as bedding in a tie

stall with one empty bed between each cow and milked twice daily at 06:30 h and 16:30 h.

Experimental silages were offered in equal amounts twice daily, 07:00 h and 17:00 h, for *ad libitum* intake, and amount of silage offered and leftovers were recorded daily. The amount of silage offered was adjusted to prompt 3–5 kg leftovers. No concentrates were offered, but 100 g of a granulated mineral-vitamin mixture (VM 2 Grøn, Vitfoss, Denmark; Ca, 160 g/kg; P, 50 g/kg; Mg, 65 g/kg; Na, 90 g/kg; S, 2 g/kg; Mn, 4000 mg/kg; Zn, 4500 mg/kg; Cu, 1500 mg/kg; Co, 25 mg/kg; I, 225 mg/kg; Se, 50 mg/kg; vitamin A, 600 IE/g; vitamin $D_3$, 190 IE/g; vitamin E, 4000 IE/kg) were offered daily on top of the silage at the morning feeding. Additionally, 20 g of vitamins with selenium (Rød Suplex Caps ADE, Vitfoss, Denmark; vitamin A, 5000 IE/g; vitamin $D_3$, 200 IE/g; vitamin E, 10,000 IE/kg; Se, 10 mg/kg) were offered at the morning feeding twice a week. The cows had free access to water from individual drinking bowls fitted with water gauges.

### 2.3. Experimental procedure and sample collection

One week prior to the experiment, the cows were adapted to eat pure grass-clover silage (368 g DM/kg; 153 g CP/kg DM; 320 g aNDFom/kg DM) without concentrates. The experiment consisted of five periods (P1-P5) and each period lasted 21 days. Silage assignment to cows was balanced over the course of the experiment in an incomplete balanced scheme. Four silages, one per cow, were fed in each period. To maximise the statistical power regarding silage DM concentration, the two silages with different DM concentrations made of the same cut were always fed in the same periods. The four spring growth silages were fed in P1 and P2, the four first regrowth silages were fed in P3 and P4 and the two first regrowth silages from farm 1 and the two spring growth silages from farm 2 were fed in P5. Thereby, there were three observations for four silages and two observations for the remaining four silages. During the initial 14 days of each period, the cows were adapted to the experimental silages, whereas the last 7 days of each period were used for sample collection.

To measure duodenal and ileal flow and faecal output, three markers, 10 g chromium(III) oxide ($Cr_2O_3$), 10 g titanium(IV) oxide ($TiO_2$) and 2 g ytterbium(III) chloride hexahydrate ($YbCl_3{\cdot}6H_2O$), were weighed out in degradable paper filter bags and placed in the rumen via the cannula on day 1–19 in each period in connection with the daily milkings (06:30 h and 16:30 h).

Twelve subsamples of duodenal and ileal chyme and faeces, respectively, were taken over a 94 h period on day 15–19 in each period to cover the diurnal variation (day 15, 10:00 h, 18:00 h; day 16, 02:00 h, 12:00 h, 20:00 h; day 17, 04:00 h, 14:00 h, 22:00 h; day 18, 06:00 h, 16:00 h, 24:00 h; day 19, 08:00 h), and each sample type were pooled within cow and period and stored in a freezer during the whole sampling period. The subsamples of duodenal (500 mL) and ileal (250 mL) chyme were collected with tube formed plastic bags mounted on the cannulas with 90° angled plastic tubes, and subsamples of faeces (350 mL) were collected when the cows defecated or by grab sampling. In the same sampling procedure, rumen fluid from the ventral part was collected through the rumen cannula using a plastic syringe and a suction strainer. Rumen fluid pH was measured immediately with a pH meter and two 8 mL subsamples were frozen until analysis.

Microbes were isolated from the rumen to estimate the duodenal flow of microbial protein. On day 19 in each period, two litres of rumen fluid from the ventral part were collected with a beaker through the rumen cannula at 11:15 h for two cows and at 12:30 h for the two remaining cows. The rumen fluid was filtered over four layers of cheesecloth into preheated isolated bottles. The bottles were transported from the barn to the laboratory and the rumen fluid was centrifuged twice in a high speed refrigerate centrifuge at 500 × *g* for 5 min (3 °C) to remove small particles and protozoa. To precipitate microbes, the supernatant was centrifuged at 17,300 × *g* for 20 min (3 °C) and the precipitate was suspended in 200 mL physiological saline water (9 g NaCl dissolved in 1.0 L ion-exchanged water) and re-centrifuged in the same way to purify the microbes. The pellet was stored frozen until freeze-drying and analysis.

On day 19 in each period, after the evening milking, the cows were moved to individual open-circuit respiration chambers (17 $m^3$) (Hellwing et al., 2012) to get acclimatised before measuring the methane ($CH_4$) production on day 20 and 21 (48 h). The chambers were located in the same barn as the cows were housed in order to reduce environmental changes, and were covered with transparent polycarbonate and placed in a square so the cows faced each other. The daily routines were identical to the rest of the feeding period and the chambers were opened for milking and feeding for about 20 min twice daily. The $CH_4$ concentration in background air (inlet air) and chamber outlet air for each chamber were measured every 12.5 min with an infrared analyser and the airflow was measured with a HFM-200 flow meter with a laminar flow element from Teledyne Hastings Instruments (Hampton, Virginia, USA). All other instruments were from Columbus Instruments (Columbus, Ohio, USA). Before the cows entered the chambers, the $CH_4$ sensor was calibrated with zero gas (nitrogen) and a span gas with 20.49% $O_2$, 4980 ppm $CO_2$, 799 ppm $CH_4$, 151 ppm $H_2$ and the remainder nitrogen (AGA A/S, Copenhagen, Denmark). The recovery rate of $CH_4$ was controlled before, during and after the experiment for each chamber and the recovery was in average 101 ± 1% (mean ± SD) for the four chambers. The $CH_4$ production, while the chambers were opened, was assumed equal to the mean of the rest of the day, as the actual measurements were deleted. Methane production was measured as the accumulated amount in litre over 24 h, corrected with the recovery rate, and reported under standard conditions (0 °C, 101.325 kPa). For further details see Hellwing et al. (2012).

Daily milk yield (two milkings) was recorded on day 16 and 17 in each period and milk samples were taken and analysed for fat, protein and lactose concentration on a Milkoscan 4000 infrared analyser (Foss Electric, Hillerød, Denmark) at Eurofins Steins Laboratorium (Vejen, Denmark). Daily water intake was recorded on day 15–18 in each period, and in each period, the cows were weighed when moved into and out of the respiration chambers, respectively.

### 2.4. Chemical analyses

Dry matter concentration of feed samples and feed residues was determined on day 15–19 by drying in a forced air oven at 60 °C for 48 h. All samples for chemical analyses were freeze-dried and ground to 1 mm in a hammer mill. Ash was determined by combustion at 525 °C for 6 h. Nitrogen was analysed by the Dumas method (Hansen, 1989) using a Vario MAX CN (Elementar Analysesysteme GmbH, Hanau, Germany), and CP was calculated as N × 6.25. The neutral detergent fibre (aNDFom) concentration was analysed according to Mertens (2002) in Fibertec M6 System (Foss Analytical, Hillerød, Denmark) including sodium sulphite and heat stable amylase treatments and corrected for ash content. Crude fat was determined by HCl hydrolysis followed by extraction with petroleum ether (Stoldt, 1952) in a Soxtec system (Foss Analytical, Hillerød, Denmark). Purines in microbes and duodenal chyme were analysed by spectrophotometry at 260 nm after precipitating with $AgNO_3$ and dissolved in excess HCl according to Zinn and Owens (1986) and with modifications according to Thode (1999). In digesta and faecal samples, chromium oxide was determined by spectrophotometry after oxidation with sodium peroxide to chromate (Schürch et al., 1950) and ytterbium was analysed using inductively coupled plasma mass spectroscopy (XSeries, ICP-MS, Thermo Fisher Scientific Germany Ltd. & Co. KG, Bonn, Germany). Background titanium oxide in silages and titanium oxide in digesta and faecal samples were measured spectrophotometrically after digestion with sulfuric acid followed by addition of hydrogen peroxide according to Myers et al. (2004) with the modification that 15 mL of 30% hydrogen peroxide were added instead of 10 mL, and before measuring the absorbance additional 5 drops hydrogen peroxide were added. Soluble N in silages was analysed by extraction in a borate-phosphate buffer (pH 6.75) at 39 °C for one hour (Åkerlind et al., 2011). *In vitro* organic matter (OM) digestibility (IVOMD) of silages was determined by incubation in rumen fluid for 48 h followed by incubation in a pepsin-HCl-solution (Tilley and Terry, 1963), ending with combustion of residues. *In vivo* OM digestibility (OMD) was calculated as 4.10 + 0.959 × IVOMD (Møller et al., 1989). The indigestible aNDFom (iNDF) concentration in silages was determined as the aNDFom residue left after 288 h (12 days) incubation of 1.5 mm milled samples in Dacron bags in the rumen of dry cows fed at maintenance (Åkerlind et al., 2011). Total sugar in silages was determined by the Luff-Schoorl method (European Community, 2012, 71/250/EEC). Acid detergent lignin (ADL) concentration in silages was determined according to ISO method 13908 (ISO, 2008). Gross energy of silages was determined using an adiabatic bomb calorimeter (Parr 6300 Oxygen Bomb Calorimeter, Parr Instrument Company, Moline, IL, USA).

Silage extracts were used to analyse silage pH and fermentation products. Water (1000 mL) was added to chopped silage (100 g) and blended for 2 × 40 s in a Waring blender (Waring 24CB10; Waring Commercial, New Hartford, CT). Two subsamples of 50 mL each were centrifuged at 2300 × *g* for 20 min (10 °C) using RPM 3200 (Hettich Rotixa 50RS) and pH was measured in the supernatant before stabilising with 5% meta-phosphoric acid. In silage extracts and rumen fluid, volatile fatty acids were analysed by gas chromatography as described by Kristensen et al. (1996). Ammonia N ($NH_3$-N) was determined using a Cobas Mira auto-analyser (Triolab A/S, Brøndby, Denmark) and a kit based on glutamate dehydrogenase (AM 1015; Randox Laboratories Ltd, Crumlin, UK). Glucose and L-lactate were determined with membrane-immobilised substrate specific oxidases using an YSI 2900 Biochemistry Analyser (YSI Inc., Yellow Springs, OH, USA). Alcohols and alcohol esters in silage extracts were determined by headspace GC–MS (Kristensen et al., 2010).

Total AA, except DAPA, tyrosine and tryptophan, in silages, microbes, duodenal and ileal chyme were determined by oxidation with performic acid and subsequent hydrolysis with hydrochloric acid (Mason et al., 1980), followed by quantitative determination of individual AA using a Biochrom B20 automated AA analyser. Serine, valine and isoleucine were corrected with a factor of 1.06 as they are acid labile and moderately prone to oxidation (Rudemo et al., 1980), and AA nitrogen (AA-N) was calculated based on the proportion of N in each single AA.

### 2.5. Calculations

Energy corrected milk yield (ECM, 3.14 MJ/kg) was calculated by the formula: ECM = 0.01 × milk yield in kg + 12.2 × milk fat in kg + 7.7 × milk protein in kg + 5.3 × milk lactose in kg (Sjaunja et al., 1990).

Duodenal and ileal DM flow and faecal DM output were calculated based on the average of the concentration of each marker in relation to daily supply. The silages contained a considerable amount of titanium oxide (Table 1), which was adjusted for. Due to the approach of forage evaluation, the flows were related to dry matter intake (DMI) and expressed as g/kg DMI.

Duodenal microbial DM flow was calculated, using purines as internal marker, as duodenal DM flow times duodenal purine concentration divided by purine concentration in rumen-isolated microbes, assuming that purines in duodenal chyme was only of microbial origin.

### 2.6. Statistical analyses

All statistical analyses were performed with R 3.3.0 (R Core Team, 2016).

Differences in chemical composition between silages (n = 8) according to farm, cut number and DM concentration were analysed by the linear regression model:

$$Y_{cdf} = \mu + \alpha d + \beta_f + \gamma_c + (\beta\gamma)_{fc} + E_{cdf}$$

where $Y_{cdf}$ is the dependent variable, $\mu$ is the overall mean, $\alpha$ is the regression coefficient for the DM concentration d, $\beta_f$ is the fixed effect of farm (f = 1 or 2), $\gamma_c$ is the fixed effect of cut number (c = 1 or 2), $(\beta\gamma)_{fc}$ is the interaction between farm and cut number and $E_{cdf}$ is the residual error assumed to be independent and normal distributed. Significance level was tested with an adjusted variance test.

**Table 1**
Chemical composition of experimental grass-clover silages.

| | Farm 1 | | | | Farm 2 | | | | Δ by 100 g/kg | | P-values | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Spring growth | | First regrowth | | Spring growth | | First regrowth | | DM increase | SE[c] | DM | Farm | Cut No. | Farm*Cut No |
| DM (g/kg fresh matter) | 283 | 644 | 322 | 427 | 492 | 660 | 377 | 725 | | | | | | |
| Titanium oxide (g/kg DM) | 0.14 | 0.13 | 0.29 | 0.40 | 0.13 | 0.10 | 0.35 | 0.12 | | | | | | |
| Ash (g/kg DM) | 84.0 | 85.1 | 126 | 139 | 89.0 | 81.9 | 120 | 88.8 | −3.53 | 3.15 | 0.343 | 0.532 | 0.037 | 0.216 |
| CP (g/kg DM) | 158 | 149 | 176 | 180 | 133 | 125 | 148 | 163 | +0.41 | 2.14 | 0.861 | 0.013 | 0.007 | 0.879 |
| Total AA (g/kg DM) | 113 | 111 | 131 | 135 | 94.7 | 94.5 | 109 | 124 | +1.61 | 1.37 | 0.325 | 0.006 | 0.002 | 0.974 |
| aNDFom (g/kg DM) | 429 | 484 | 390 | 390 | 490 | 480 | 364 | 381 | +8.25 | 3.99 | 0.131 | 0.795 | 0.006 | 0.102 |
| iNDF (g/100 g aNDFom) | 11.0 | 10.7 | 11.8 | 11.5 | 9.63 | 10.5 | 16.1 | 12.5 | −0.43 | 0.32 | 0.267 | 0.342 | 0.131 | 0.116 |
| ADL (g/kg DM) | 18.6 | 19.9 | 20.0 | 21.1 | 19.5 | 25.4 | 27.9 | 25.4 | +0.25 | 0.70 | 0.744 | 0.102 | 0.184 | 0.525 |
| Crude fat (g/kg DM) | 33.1 | 23.5 | 33.0 | 32.1 | 24.9 | 20.5 | 34.1 | 25.5 | −2.51 | 0.20 | 0.001 | 0.633 | 0.023 | 0.026 |
| Total sugar (g/kg DM) | 47.2 | 123 | 14.2 | 43.2 | 107 | 145 | 73.4 | 164 | +23.5 | 1.40 | < 0.001 | 0.043 | 0.123 | 0.021 |
| Glucose (g/kg DM) | 5.97 | 34.8 | 5.47 | 14.4 | 27.4 | 36.6 | 19.7 | 40.7 | +6.95 | 0.61 | 0.001 | 0.046 | 0.318 | 0.291 |
| N (g/100 g total N) | | | | | | | | | | | | | | |
| Soluble N | 63.2 | 42.4 | 56.1 | 52.2 | 59.2 | 39.8 | 59.1 | 29.5 | −7.40 | 1.14 | 0.007 | 0.248 | 0.089 | 0.796 |
| $NH_3$-N | 5.06 | 1.94 | 5.97 | 5.83 | 3.47 | 2.04 | 6.09 | 1.43 | −1.03 | 0.17 | 0.009 | 0.883 | 0.068 | 0.482 |
| AA-N | 60.1 | 63.3 | 62.2 | 63.6 | 61.7 | 64.4 | 62.6 | 65.1 | +0.90 | 0.15 | 0.010 | 0.897 | 0.025 | 0.328 |
| Gross energy (MJ/kg DM) | 18.0 | 17.7 | 17.1 | 17.0 | 17.4 | 17.5 | 17.1 | 17.6 | +0.03 | 0.07 | 0.717 | 0.736 | 0.170 | 0.141 |
| OMD (g/kg OM)[a] | 788 | 772 | 783 | 780 | 772 | 776 | 775 | 776 | −1.81 | 1.49 | 0.310 | 0.408 | 0.912 | 0.839 |
| pH | 4.17 | 5.85 | 4.04 | 4.20 | 5.50 | 5.56 | 4.28 | 5.48 | +0.36 | 0.08 | 0.017 | 0.587 | 0.035 | 0.984 |
| L-Lactate (g/kg DM) | 23.5 | 0.69 | 39.2 | 25.4 | 2.14 | 0.39 | 24.3 | 0.40 | −6.29 | 1.24 | 0.015 | 0.162 | 0.020 | 0.503 |
| Acetate (g/kg DM) | 10.9 | ND[b] | 19.9 | 12.8 | 1.70 | ND | 12.6 | ND | −3.22 | 0.59 | 0.012 | 0.234 | 0.019 | 0.367 |
| Butyrate (g/kg DM) | 1.01 | ND | ND | ND | ND | ND | 1.95 | ND | −0.24 | 0.05 | 0.022 | 0.449 | 0.683 | 0.032 |
| Ethanol (g/kg DM) | 43.5 | 17.3 | 9.59 | 5.97 | 61.3 | 20.5 | 14.5 | 8.04 | −6.50 | 3.66 | 0.174 | 0.165 | 0.027 | 0.894 |
| Propanol (mg/kg DM) | 100 | 28.2 | 75.1 | 19.9 | 40.9 | 28.9 | 28.0 | 16.6 | −13.0 | 6.42 | 0.136 | 0.637 | 0.235 | 0.748 |
| Butanol (mg/kg DM) | 146 | 6.57 | 84.4 | 59.4 | 52.9 | 26.4 | 232 | 44.9 | −42.1 | 6.88 | 0.009 | 0.154 | 0.536 | 0.040 |
| Ethyl acetate (mg/kg DM) | 1149 | 43.5 | 431 | 229 | 275 | 60.9 | 529 | 21.0 | −21.8 | 46.4 | 0.018 | 0.777 | 0.312 | 0.135 |
| Propyl acetate (mg/kg DM) | 3.54 | ND | 5.40 | 1.51 | ND | ND | 1.62 | ND | −0.77 | 0.38 | 0.138 | 0.351 | 0.434 | 0.866 |

[a] *In vivo* organic matter digestibility calculated as 4.10 + 0.959 × *in vitro* organic matter digestibility (Møller et al., 1989).
[b] Not detected, entered in the analysis with the value zero.
[c] Standard error for the change for each increase in silage DM concentration of 100 g/kg.

The linear effect of silage DM concentration on the various animal measurements (n = 20) were analysed using the *lmer* function from the *lme4* package (Bates et al., 2015) and the following random regression model fitted with REML:

$$Y_{acdf} = \mu + \alpha d + A_a + B_{cf} + C_{cf}d + E_{acdf}$$

where $Y_{acdf}$ is the dependent variable, $\mu$ is the overall mean, α is the regression coefficient for silage DM concentration d, $A_a$ is the random effect of cow (a = 1–4), $B_{cf}$ is the random effect of cut number within farm (c = 1 or 2 and f = 1 or 2), $C_{cf}$ is the random regression coefficient for silage DM concentration d within cut number for each farm and $E_{acdf}$ is the residual error assumed to be independent and normal distributed. The ileal CP and AA flow for one cow in one period were discarded in the analysis, due to a dubious sample and outlier results. In the analysis of milk yield and milk composition, period was added to the model as continuous variable to test for persistency, giving the following random regression model:

$$Y_{acdfp} = \mu + \alpha d + \beta p + A_a + B_{cf} + C_{cf}d + E_{acdfp}$$

where $\beta$ is the regression coefficient for period number p. The predicted value of a given dependent variable at the average silage DM concentration (488 g/kg), the change in the dependent variable with each increase in silage DM concentration of 100 g/kg and the standard error (SE) for the change are presented in the tables. The effect of the regression coefficient for silage DM concentration was tested with parametric bootstrapping with 10,000 repetitions. Statistical significance was determined by $P \leq 0.05$ and tendencies by $P \leq 0.10$.

## 3. Results

### 3.1. Silages

Silage DM concentration ranged from 283 to 725 g/kg (Table 1). The concentration of total sugar ($P < 0.001$) and glucose ($P = 0.001$) increased with increased DM concentration and was highest in silages from farm 2. Ash, CP, total AA, aNDFom and ADL concentrations in DM and iNDF as proportion of aNDFom were not affected by DM concentration ($P > 0.1$), but ash, CP, total AA and aNDFom concentrations in DM were affected by cut number and CP and total AA concentrations in DM were further affected by farm. Concentration of crude fat decreased ($P = 0.001$) with increased DM concentration and was further affected by cut number and interacted with farm and cut number. Soluble N and $NH_3$-N as proportion of total N decreased ($P = 0.007$ and 0.009, respectively), whereas AA-N as proportion of total N increased ($P = 0.010$) with increased DM concentration. Concentration of the fermentation products L-lactate ($P = 0.015$), acetate ($P = 0.012$) and butyrate ($P = 0.022$) decreased with increased DM concentration and regrowth silages had a higher concentration of L-lactate and acetate than spring growth silages. Silage pH increased ($P = 0.017$) with increased DM concentration, whereas the concentration of butanol ($P = 0.009$) and ethyl acetate ($P = 0.018$) decreased with increased DM concentration. Concentration of ethanol, propanol and propyl acetate decreased numerically with increased DM concentration. Silage gross energy concentration and OMD were unaffected by DM concentration ($P > 0.3$, Table 1).

### 3.2. Intake and production

Body weight, DMI and milk production were stable with a minor decrease throughout the experimental period. Body weight was 556 ± 31 (mean ± SD) kg in P1 and 543 ± 21 kg in P5, DMI was 12.4 ± 2.6 kg in P1 and 11.5 ± 2.1 kg in P5 and ECM yield was 16.4 ± 3.4 kg in P1 and 12.7 ± 2.3 kg in P5, respectively. The decline in milk yield corresponded to the decline expected with normal persistency over a 105-day period.

Average daily intake of DM (12.4 kg/d), OM (11.1 kg/d), aNDFom (5.26 kg/d), CP (1.92 kg/day), AA (1.42 kg/d) and crude fat (0.36 kg/d) was unaffected by silage DM concentration ($P > 0.1$, Table 2). Total intake of water was also unaffected ($P = 0.893$) by silage DM concentration, but water intake through the feed decreased ($P = 0.021$) with increased silage DM concentration, by which intake of tap water increased equivalently ($P = 0.035$, Table 2).

Milk yield, milk composition and ECM yield were unaffected by silage DM concentration ($P > 0.1$, Table 2).

### 3.3. Duodenal flow

Total duodenal flow of OM in g/kg DMI increased ($P = 0.004$, Table 3) and total duodenal flow of crude fat in g/kg DMI decreased ($P = 0.012$) with increased silage DM concentration. When increasing silage DM concentration total duodenal flow of AA in g/kg DMI tended to increase ($P = 0.079$), whereas total duodenal flow of DM, aNDFom and CP in g/kg DMI was not affected (Table 3). Duodenal microbial CP flow in g/kg DMI increased ($P = 0.034$, Table 3) with increased silage DM concentration, whereas duodenal microbial flow of DM, OM and AA in g/kg DMI tended to increase ($P = 0.064$, 0.051 and 0.059, respectively). Duodenal flow of OM in g/kg DMI from undegraded feed and endogenous sources increased ($P = 0.028$), whereas duodenal flow of AA in g/kg DMI from undegraded feed and endogenous sources tended to increase ($P = 0.070$). Silage DM concentration did not affect duodenal flow of DM and CP in g/kg DMI from undegraded feed and endogenous sources.

**Table 2**
Nutrient and water intake, and milk production predicted at average silage DM concentration (488 g/kg) and the change with each increase in silage DM concentration of 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[a] | P-value |
|---|---|---|---|---|
| Intake (kg/d) | | | | |
| DM | 12.4 | +0.26 | 0.39 | 0.588 |
| OM | 11.1 | +0.27 | 0.38 | 0.556 |
| aNDFom | 5.26 | +0.23 | 0.12 | 0.163 |
| CP | 1.92 | +0.04 | 0.08 | 0.648 |
| AA | 1.42 | +0.06 | 0.06 | 0.441 |
| Crude fat | 0.36 | −0.02 | 0.01 | 0.182 |
| Intake (L/d) | | | | |
| Feed water | 15.3 | −5.37 | 1.13 | 0.021 |
| Tap water | 55.9 | +5.69 | 1.39 | 0.035 |
| Total water | 71.4 | −0.34 | 1.92 | 0.893 |
| Milk production | | | | |
| Yield (kg/d) | 14.5 | +0.07 | 0.54 | 0.907 |
| Fat (g/kg) | 42.8 | −0.47 | 0.39 | 0.520 |
| Protein (g/kg) | 32.3 | +0.46 | 0.42 | 0.360 |
| Lactose (g/kg) | 44.6 | +0.20 | 0.14 | 0.135 |
| ECM (kg/d) | 14.8 | +0.07 | 0.52 | 0.890 |

[a] Standard error for the change for each increase in silage DM concentration of 100 g/kg.

**Table 3**
Duodenal nutrient flow and amount of AA digested in the small intestine predicted at average silage DM concentration (488 g/kg) and the change with each increase in silage DM concentration of 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[a] | P-value |
|---|---|---|---|---|
| Total duodenal flow (g/kg DMI) | | | | |
| DM | 722 | −3.12 | 10.2 | 0.759 |
| OM | 493 | +15.7 | 3.43 | 0.004 |
| aNDFom | 98.6 | −1.87 | 2.79 | 0.655 |
| CP | 189 | +5.44 | 2.30 | 0.119 |
| AA | 145 | +5.63 | 1.98 | 0.079 |
| Crude fat | 42.6 | −3.00 | 0.64 | 0.012 |
| Duodenal microbial flow (g/kg DMI) | | | | |
| DM | 144 | +10.8 | 3.62 | 0.064 |
| OM | 103 | +6.38 | 2.14 | 0.051 |
| CP | 65.4 | +3.18 | 0.95 | 0.034 |
| AA | 49.9 | +2.12 | 0.77 | 0.059 |
| Duodenal feed + endogenous flow (g/kg DMI) | | | | |
| DM | 574 | −11.7 | 11.9 | 0.389 |
| OM | 391 | +8.16 | 3.04 | 0.028 |
| CP | 123 | +3.10 | 1.82 | 0.202 |
| AA | 95.2 | +4.29 | 1.60 | 0.070 |
| AA digested in small intestine (g/kg DMI) | 99.4 | +5.59 | 1.45 | 0.024 |

[a] Standard error for the change for each increase in silage DM concentration of 100 g/kg.

### 3.4. Digestibility

Apparent rumen digestibility of DM, aNDFom and crude fat was unaffected by silage DM concentration ($P > 0.1$, Table 4), whereas apparent rumen digestibility of OM ($P = 0.016$), CP ($P = 0.041$) and AA ($P = 0.020$) decreased with increased silage DM concentration. Rumen true digestibility of CP and AA decreased by 0.044 ($P = 0.016$) and 0.040 ($P = 0.041$), respectively, with each increase in silage DM concentration of 100 g/kg. Apparent small intestinal digestibility of DM, OM and crude fat was unaffected by silage DM concentration ($P > 0.1$, Table 4), whereas apparent small intestinal digestibility of AA increased by 0.013 ($P = 0.034$) with each increase in silage DM concentration of 100 g/kg. Increasing silage DM concentration tended to increase the apparent small intestinal digestibility of CP ($P = 0.060$). Apparent large intestinal digestibility was not affected by silage DM concentration for any of the measured nutrients (Table 4). For crude fat, apparent total tract digestibility decreased ($P = 0.039$) with increased silage DM concentration, whereas apparent total tract digestibility of DM, OM, CP and aNDFom was unaffected by silage DM concentration.

**Table 4**
Nutrient digestibility in the rumen, small intestine, large intestine and total tract, predicted at average silage DM concentration (488 g/kg) and the change with each increase in silage DM concentration of 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[a] | P-value |
|---|---|---|---|---|
| Apparent rumen digestibility | | | | |
| DM | 0.278 | +0.003 | 0.010 | 0.759 |
| OM | 0.451 | −0.012 | 0.004 | 0.016 |
| aNDFom | 0.761 | +0.003 | 0.005 | 0.598 |
| CP | −0.233 | −0.049 | 0.015 | 0.041 |
| AA | −0.291 | −0.048 | 0.018 | 0.020 |
| Crude fat | −0.518 | −0.034 | 0.026 | 0.194 |
| True rumen digestibility | | | | |
| CP | 0.186 | −0.044 | 0.016 | 0.016 |
| AA | 0.156 | −0.040 | 0.015 | 0.041 |
| Apparent small intestinal digestibility | | | | |
| DM | 0.480 | −0.009 | 0.009 | 0.373 |
| OM | 0.436 | +0.004 | 0.003 | 0.218 |
| CP | 0.628 | +0.010 | 0.004 | 0.060 |
| AA | 0.686 | +0.013 | 0.004 | 0.034 |
| aNDFom | −0.195 | −0.048 | 0.018 | 0.084 |
| Crude fat | 0.649 | +0.001 | 0.009 | 0.891 |
| Apparent large intestinal digestibility | | | | |
| DM | 0.190 | +0.001 | 0.008 | 0.884 |
| OM | 0.120 | −0.003 | 0.010 | 0.810 |
| CP | 0.138 | −0.007 | 0.008 | 0.389 |
| aNDFom | 0.177 | −0.005 | 0.018 | 0.988 |
| Crude fat | −0.021 | −0.010 | 0.011 | 0.440 |
| Apparent total tract digestibility | | | | |
| DM | 0.699 | −0.001 | 0.006 | 0.935 |
| OM | 0.727 | −0.005 | 0.004 | 0.236 |
| CP | 0.602 | −0.014 | 0.006 | 0.105 |
| aNDFom | 0.770 | −0.001 | 0.006 | 0.820 |
| Crude fat | 0.472 | −0.031 | 0.010 | 0.039 |

[a] Standard error for the change for each increase in silage DM concentration of 100 g/kg.

### 3.5. Metabolisable protein

The combined effect of a tendency towards an increased duodenal flow of AA per kg DMI and an increased digestibility of AA in the small intestine increased the amount of AA digested in the small intestine by 5.59 g/kg DMI with each increase in silage DM concentration of 100 g/kg ($P = 0.024$, Table 3).

### 3.6. Rumen fluid

Short chain fatty acid (SCFA) concentration and pH in rumen fluid were not affected by silage DM concentration ($P > 0.5$, Table 5), whereas the total concentration of branched-chain SCFA decreased ($P = 0.008$) with increased silage DM concentration. Isovalerate as proportion of total SCFA decreased ($P = 0.009$) and isobutyrate as proportion of total SCFA tended to decrease ($P = 0.055$) with increased silage DM concentration (Table 5), whereas the concentration of the other SCFA as proportion of total SCFA was unaffected ($P > 0.2$). Concentration of $NH_3$-N in rumen fluid decreased ($P = 0.006$) with increased silage DM concentration. The decreased concentration of isobutyrate, isovalerate and $NH_3$-N was consistent over the day (data not shown).

### 3.7. Methane production

The cows produced on average 414 L $CH_4$/d and the $CH_4$ production was not affected by silage DM concentration ($P = 0.193$, Table 6), neither when expressed per kg DMI or as percentage of gross energy intake.

## 4. Discussion

### 4.1. Silages

The ratios among silage fermentation products and the absence of or low concentration of butyrate indicated that all eight silages were well preserved (McDonald et al., 1991). The planned silage DM concentrations of 350 and 700 g/kg were not fully achieved, but longer wilting increased the DM concentration for all four cuts, and resulting DM concentrations covered the range from 283 to

**Table 5**
Rumen pH, SCFA and $NH_3$-N concentration (average of 12 diurnal samples) predicted at average silage DM concentration (488 g/kg) and the change with each increase in silage DM concentration of 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[a] | P-value |
|---|---|---|---|---|
| pH | 6.62 | 0.00 | 0.02 | 0.952 |
| Total SCFA (mmol/L) | 100 | −1.72 | 2.44 | 0.522 |
| Total branched-chain SCFA (mmol/L) | 2.72 | −0.53 | 0.08 | 0.008 |
| SCFA (mol/100 mol total SCFA) | | | | |
| L-Lactate | 0.39 | −0.02 | 0.05 | 0.412 |
| Acetate | 66.2 | +0.39 | 0.62 | 0.539 |
| Propionate | 17.8 | +0.40 | 0.58 | 0.544 |
| Isobutyrate | 0.90 | −0.09 | 0.02 | 0.055 |
| Butyrate | 10.2 | +0.06 | 0.21 | 0.984 |
| Isovalerate | 1.78 | −0.39 | 0.06 | 0.009 |
| Valerate | 1.88 | −0.06 | 0.04 | 0.232 |
| Caproate | 0.95 | −0.16 | 0.11 | 0.235 |
| $NH_3$-N (mmol/L) | 5.39 | −0.92 | 0.21 | 0.006 |

[a] Standard error for the change for each increase in silage DM concentration of 100 g/kg.

**Table 6**
Methane production predicted at average silage DM concentration (488 g/kg) and the change with each increase in silage DM concentration of 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[b] | P-value |
|---|---|---|---|---|
| $CH_4$ production | | | | |
| L/d | 414 | +16.7 | 9.23 | 0.193 |
| L/kg DMI[a] | 31.2 | −0.06 | 0.28 | 0.869 |
| % of gross energy intake | 7.09 | −0.02 | 0.07 | 0.877 |

[a] Dry matter intake during $CH_4$ measurements.
[b] Standard error for the change for each increase in silage DM concentration of 100 g/kg.

725 g/kg. Crude protein, total AA, total sugar and glucose concentrations varied between farms, and ash, CP, total AA, aNDFom and crude fat concentrations varied between cut numbers, by which the intended variation in chemical composition between the four cuts was achieved. Overall, concentrations of ash, CP, aNDFom, iNDF and gross energy were unaffected by increased DM concentration, indicating that leaf material was not lost in significant amount in the field during wilting. The increased concentration of sugar, glucose and AA-N in total N and the decreased concentration of soluble N and $NH_3$-N in total N revealed that microbial fermentation during the ensiling process diminished with increased DM concentration, which also was substantiated by the lower accompanying concentration of fermentation products. The changed distribution of nitrogen components supported the contention that higher DM concentration reduces proteolysis (Slottner and Bertilsson, 2006). The observed lower concentration of crude fat in silages with higher DM concentrations might be related to oxidation of unsaturated fatty acids during wilting, as DM concentration does not affect total fatty acid concentration during ensiling (van Ranst et al., 2009). van Ranst et al. (2009) showed that a higher DM concentration in grass herbage reduces lipolysis during ensiling, giving a higher concentration of triglycerides and a lower concentration of free fatty acids in grass silage with higher DM concentration. Whether the distribution of the lipid fractions varied in the silages in the current study was not analysed, but it was assumed, that lipolysis was reduced in the same way as proteolysis.

### 4.2. Silage and water intake

Dry matter intake was not affected by silage DM concentration, which is in agreement with other studies (Campbell and Buchanan-Smith, 1991; Verbic et al., 1999; Gordon et al., 2000; Purwin et al., 2009). Huhtanen et al. (2007) concluded that DM concentration is important to predict DMI, but most studies included in their analysis compared direct cut silage with wilted silage or silage with hay, and not silages wilted to different DM concentrations. It is widely documented, that DMI of wilted silage is higher than that of direct cut silage (Castle and Watson, 1984; Gordon and Peoples, 1986; Peoples and Gordon, 1989; Patterson et al., 1998), but nothing indicates that DMI should differ between silages wilted to different DM concentrations if above 300 g/kg.

Water intake through feed decreased, when cows were offered silage with higher DM concentration. However, intake of tap water compensated the decreased feed water intake fully, by which total water intake was unaffected. Castle and Watson (1970) have reported a similar full compensation of water intake in cows fed grass silage with 205 and 318 g DM/kg, respectively. This indicates that requirement for water is highly regulated and that water source is unimportant.

### 4.3. Metabolisable protein

The increased amount of AA digested in the small intestine per kg DMI proved that the MP concentration in the grass-clover silage

increased with increased silage DM concentration. Merchen and Satter (1983) reported an AA digestion in the small intestine of 1075, 1284 and 1517 g/d in dairy cows fed alfalfa silage with 290, 400 and 660 g DM/kg, respectively, but with no significant differences. In the current study, a numerical, but not significant, increase was observed as well when analysing the AA digestion in the small intestine in g/d. Recalculation of the figures from Merchen and Satter (1983) showed an increase in the amount of AA digested in the small intestine of 5.17 g/kg DMI when increasing silage DM concentration by 100 g/kg, which corresponds well to the observed increase of 5.59 g/kg DMI in the current study. This indicates that pre-wilting to higher DM concentration affects the protein value in the same way independently of forage species provided efficient pre-wilting.

The higher digestion of AA in the small intestine was caused by an increased digestibility of AA in the small intestine as well as an increased duodenal flow of microbial AA and AA from feed and endogenous sources, the two latter however only as tendencies. The figures in the current study indicated that rumen microbial synthesis increased when increasing silage DM concentration, as the duodenal flow of microbial CP per kg DMI increased and the duodenal flow of microbial DM, OM and AA per kg DMI tended to increase. This is in agreement with Verbic et al. (1999) who calculated, based on purine derivatives excretion in urine, a higher microbial nitrogen supply in sheep fed grass silage wilted to 521 g DM/kg compared to grass silage wilted to 432 g DM/kg. Conversely, Merchen and Satter (1983) did not find any differences in the daily microbial N flow at the duodenum in cows fed alfalfa silages wilted to different DM concentrations. However, wilting of grass prior to ensiling increases rumen microbial synthesis, compared to direct cut silage (Narasimhalu et al., 1989; Teller et al., 1992; Yan et al., 1998). The increased microbial synthesis is in agreement with the theoretical assumption, that a higher concentration of water soluble carbohydrates in silage with increased DM concentration supports a higher microbial growth (Chamberlain, 1987). The tendency towards an increased duodenal flow of AA from feed and endogenous sources was probably caused by an increase in AA from RUP, as the rumen digestibility of AA decreased when increasing silage DM concentration. Further it is assumed, that the endogenous flow of nutrients is constant independent of silage DM concentration, as the total duodenal DM flow was not affected (Larsen et al., 2000). The observed lower degradation of feed protein in the rumen reflected the lower concentration of soluble N in the silages with higher DM concentration and is in agreement with several *in situ* measurements concerning the effect of silage DM concentration on rumen protein degradation (van Vuuren et al., 1990; Tamminga et al., 1991; Verbic et al., 1999; Aufrere et al., 2003; Edmunds et al., 2014). Further, the current *in vivo* results support *in situ* studies on the current silages showing a decreased effective rumen protein degradability with increased silage DM concentration (Johansen and Weisbjerg, 2016). The reduced degradation of AA in the rumen was also reflected in the composition of rumen SCFA, where isovalerate proportion decreased and isobutyrate proportion tended to decrease with increased silage DM concentration, as branched-chain SCFA are products from breakdown of branched-chain AA (El-Shazly, 1952). Campbell and Buchanan-Smith (1991) did not find any significant decrease in branched-chain SCFA concentrations in the rumen of mid-lactating Holstein cows fed alfalfa-grass silages with different DM concentrations; however, they observed a numerical decrease for isobutyrate when increasing silage DM concentration. In the current study, a lower concentration of $NH_3$-N was observed in the rumen fluid when increasing silage DM concentration, which is in agreement with results from Aufrere et al. (2003), who found a lower $NH_3$-N concentration in the rumen of sheep fed grass silage with 580 g DM/kg compared to 420 g DM/kg. Likewise, Merchen and Satter (1983) found a lower concentration of $NH_3$-N in the rumen of dairy cows fed alfalfa silage with 660 g DM/kg compared to 290 and 400 g DM/kg, respectively. The lower concentration of $NH_3$-N in rumen fluid indicates either a lower rumen degradation of feed protein, a higher microbial synthesis or both (Tan and Murphy, 2004). In agreement with Campbell and Buchanan-Smith (1991), the total concentration of SCFA in rumen fluid was not affected by silage DM concentration in the current study, neither was rumen pH.

### 4.4. Digestibility of nutrients

Increasing silage DM concentration did not affect rumen, hindgut and total tract aNDFom digestibility. This is also emphasised by $CH_4$ production and proportion of acetate in the rumen being unaffected by silage DM concentration. A negative aNDFom digestibility was observed in the small intestine, which has been observed in other studies too (Stensig and Robinson, 1997; Lund et al., 2007; Brask et al., 2013). A higher iNDF to aNDFom ratio in duodenal chyme compared to ileal chyme (data not shown) indicated that the observed negative aNDFom digestibility was not a marker issue.

The apparent rumen CP digestibility was negative, probably due to urea recycling, and became more negative with increasing silage DM concentration. The transfer of urea to the rumen is negatively correlated to rumen ammonia concentration (Kennedy and Milligan, 1980; Røjen et al., 2008), which can explain the higher nitrogen recycling when increasing silage DM concentration, as $NH_3$-N concentration in the rumen decreased with increased silage DM concentration.

Negative crude fat digestibility was observed in rumen and hindgut probably due to microbial *de novo* fatty acids synthesis (Weisbjerg et al., 1992). The apparent total tract digestibility of crude fat decreased with increased silage DM concentration, even though silage DM concentration not affected the crude fat digestibility in the different segments of the gastro intestinal tract. Despite the previous stated assumption regarding altered distribution of lipid fractions between silages with different DM concentrations, silage DM concentration was not expected to influence crude fat digestibility. Triglycerides are rapidly hydrolysed by microbial lipases and free unsaturated fatty acids are hydrogenated to saturated fatty acids when entering the rumen, by which the lipids leaving the rumen will be fairly identical independent of source (Jenkins, 1993). The negative change in duodenal crude fat flow per kg DMI with each increase in silage DM concentration of 100 g/kg corresponded the change in crude fat concentration in the silages, even though it was not reflected in daily crude fat intake. Therefore, if the microbial *de novo* fatty acids synthesis is equal among silages, the apparent crude fat digestibility will decrease, when decreasing input of crude fat.

### 4.5. Milk production

Despite an increased digestion of AA in the small intestine with increased silage DM concentration, no impact on milk production was observed. The cows used in the experiment were in late lactation and low yielding, by which milk production probably not was limited by available AA. In normal and high yielding cows, a positive response in milk yield is expected when increasing MP intake (Huhtanen and Hristov, 2009), and especially in early lactation supply of MP is important for milk production (Larsen et al., 2014). Therefore, silage DM concentration will probably affect the milk yield if fed to cows expected to respond on MP supply.

## 5. Conclusion

Increased DM concentration in grass-clover silage, obtained by extended pre-wilting, increased the amount of AA digested in the small intestine in lactating dairy cows, due to reduced rumen degradation of feed protein, increased rumen microbial synthesis and increased small intestinal digestibility of AA. Silage DM concentration did not affect aNDFom digestibility and $CH_4$ production.

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## 5.4 Paper IV – Amino acid profile of metabolisable protein in lactating dairy cows is affected by dry matter concentration in grass-clover silage

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# Amino acid profile of metabolisable protein in lactating dairy cows is affected by dry matter concentration in grass-clover silage

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## Abstract

Our previous study showed that supply of metabolisable protein (MP) to lactating dairy cows increased with increasing dry matter (DM) concentration in grass-clover silage. Therefore, the aim of this study was to examine how amino acid (AA) profile of MP was affected by silage DM concentration. Eight grass-clover silages with DM concentrations ranging from 283 to 725 g/kg were fed *ad libitum* to four multi-fistulated dairy cows in an incomplete balanced scheme over five periods. Individual AA were analysed in silages, in microbes isolated from the rumen, and in duodenal and ileal chyme, respectively. Silage DM concentration affected silage AA profile, as proportions of arginine and proline increased and proportions of alanine, histidine, isoleucine, lysine, methionine, ornithine, serine, threonine and valine decreased with increased silage DM concentration. Crude protein (CP) and AA concentrations in DM and AA concentration in CP in microbial matter were not affected by silage DM concentration, but serine proportion in microbial AA increased and valine proportion in microbial AA decreased with increased silage DM concentration. In total duodenal AA profile, histidine proportion decreased, lysine proportion tended to decrease and glutamate proportion tended to increase with increased silage DM

concentration, mostly driven by changes in duodenal feed/endogenous AA profile. Small intestinal digestibility and absolute amount digested of all individual AA increased, either numerically or significantly, when increasing silage DM concentration. In the profile of digested AA, lysine proportion decreased, histidine proportion tended to decrease and glutamate proportion tended to increase. In conclusion, AA profile of MP is affected unfavourably by higher silage DM concentration. Based merely on total MP supply, a lower production response than expected will probably appear when increasing silage DM concentration, as either histidine or lysine often are the first limiting AA in grass based diets.

**Keywords:** pre-wilting, metabolizable protein, forage, *in vivo*; amino acid

**Abbreviations:** AA, amino acids; AA-N, amino acid nitrogen; aNDFom, neutral detergent fibre assayed with heat stable amylase and expressed exclusive of residual ash; CP, crude protein; DM, dry matter; DMI, dry matter intake; MP, metabolisable protein; $NH_3$-N, ammonia nitrogen; OM, organic matter; RUP, rumen undegraded feed protein; SE, standard error

## 1. Introduction

Supply of metabolisable protein (MP) is an important parameter in feed evaluation systems for dairy cows (Volden and Nielsen, 2011), as MP is one of the major factors affecting milk production. Total MP supply to dairy cows is a combination of rumen undegraded feed protein (RUP), rumen microbial protein synthesis, endogenous protein reaching the small intestine and small intestinal digestibility of true protein. However, supply of individual amino acids (AA) is also important, as a deficiency of essential AA (i.e. not synthesised by cows) can limit milk production, even though total MP supply is sufficient. The AA profile of digestible RUP can be very different

from the AA profile of the original diet, as rumen degradation of feed protein and digestibility of RUP in the small intestine differ among feedstuffs (Erasmus et al., 1994); and within feedstuffs, rumen degradation and small intestinal digestibility of individual AA differ (Skórko-Sajko et al., 1994; Lund et al., 2008). Thereby, composition and degradation of feed AA can affect the composition of digested AA and thus milk production, even though microbial protein constitutes a large part of total MP (Allen, 1996).

Recently, Johansen et al. (2017) showed that MP supply to lactating dairy cows increases when grass-clover silage is wilted to a higher dry matter (DM) concentration before ensiling. During wilting, proteolysis occurs and liberated AA are metabolised in varying degree. Simultaneously, proline is synthesised (Kemble and MacPherson, 1954), resulting in a modified AA profile for the final silage influenced by DM concentration and wilting rate (Edmunds et al., 2014). A change in silage AA profile or in rumen degradation of individual AA may affect AA profile of RUP, and thereby the composition of AA digested in the small intestine. Therefore, the objective of this study was to examine how AA composition of MP supplied to lactating dairy cows is affected by increased DM concentration in grass-clover silage obtained by extended pre-wilting.

## 2. Materials and methods

### *2.1 Forages, animals, feeding and experimental procedure*

Forage production, experimental procedure and sample collection are described in detail by Johansen et al. (2017). Briefly, the spring growth and the first regrowth of two grass-clover swards located at two Danish organic farms, respectively, were mown and wilted in 2013. Half of the herbage in each cut was planned to achieve a DM concentration of 350 g/kg, whereas the DM concentration was planned to 700 g/kg for the remaining herbage as a result of prolonged wilting, giving in total eight silages. After wilting, the herbages were baled, wrapped with plastic and

ensiled without additives. Bales were transported to AU Foulum, Tjele, Aarhus University, and to ensure uniformity and quality of the silages, bales of the same silage were mixed and re-packed in vacuum bags containing 7-15 kg silage before the feeding experiment.

For the feeding experiment, four lactating Danish Holstein cows, 216 (SD = 23) days from calving with an initial body weight at 551 (SD = 33) kg, were used. Two cows were in first lactation and two cows were in second lactation. All cows were fitted with cannulas in rumen, duodenum and ileum. Cows were fed *ad libitum* with silages offered in equal amounts twice daily at 07:00 h and 17:00 h. During morning feeding, the amount offered was adjusted to achieve 3-5 kg daily residues. The cows did not receive any concentrate, but a mineral-vitamin mixture was offered daily (100 g/d). Silages were assigned to cows in an incomplete balanced scheme over five periods, each of 21 days duration. In each period, four different silages were fed, one to each cow, but the two silages made of the same cut differing in DM concentration were always fed in the same periods to maximise statistical power regarding DM concentration. With this experimental scheme, three observations were obtained for four silages, and two observations were obtained for the remaining four silages.

Digestions markers (titanium(IV) oxide, chromium(III) oxide and ytterbium(III) chloride hexahydrate) were placed in the rumen via the cannula twice daily, 6:30 h and 16:30 h, concurrent with daily milkings, to measure flow of duodenal and ileal chyme. In the last week of each period, duodenal and ileal chyme were sampled twelve times over 94 h to cover every second hour of the day. The twelve subsamples within chyme type were pooled continuously during sampling and stored frozen until chemical analysis.

Two litres of rumen fluid were collected from the ventral part once in the last week of each period to isolate microbes. After filtration through cheesecloth, the rumen fluid was centrifuged twice (500 × g, 5 min, 3 °C) to remove small particles and protozoa, followed by additional centrifugation

(17300 × g, 20 min, 3 °C) to precipitate microbes. The pellet was suspended in saline and re-centrifuged to purify the microbes. Afterwards, the pellet was frozen until chemical analysis. For further details, see Johansen et al. (2017).

*2.2 Chemical analyses*

Feed samples and feed residues were dried (60 °C, 48 h) to determine DM concentration. All samples were freeze-dried and ground to 1 mm before chemical analyses. Combustion (525 °C, 6 h) was used to determine ash concentration. Crude protein (CP) was calculated as N × 6.25, where N was determined according to the Dumas method (Hansen, 1989). Silage samples were boiled in a neutral detergent solution added sodium sulphite and heat stable amylase followed by combustion to determine the ash-corrected concentration of neutral detergent fibre (aNDFom) (Mertens, 2002). Extraction of silage samples in a borate-phosphate buffer (pH 6.75, 39°C, 1 h) was used to determine soluble N (Åkerlind et al., 2011). Ammonia N ($NH_3$-N) was determined in silage extracts (chopped silage blended with water and centrifuged) stabilised with 5% meta-phosphoric acid using a kit based on glutamate dehydrogenase (AM 1015; Randox Laboratories Ltd, Crumlin, UK). According to Zinn and Owens (1986) and with modifications according to Thode (1999), microbes and duodenal chyme were precipitated with $AgNO_3$ and dissolved with HCl in surplus, after which purines were analysed spectrophotometrically. Duodenal and ileal samples were oxidised with sodium peroxide to chromate, after which chromium oxide was determined spectrophotometrically (Schürch et al., 1950). Further, duodenal and ileal samples were digested with sulfuric acid followed by addition of hydrogen peroxide, after which titanium oxide was measured spectrophotometrically (Myers et al., 2004). Ytterbium was analysed using inductively coupled plasma mass spectroscopy (XSeries, ICP-MS, Thermo Fisher Scientific Germany Ltd. & Co. KG, Bonn, Germany). Silages samples, microbes, and duodenal and ileal chyme were oxidised with

performic acid and hydrolysed with hydrochloric acid (Mason et al., 1980) followed by quantitative determination of alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine and valine using a Biochrom B20 automated AA analyser. The acid labile AA (serine, valine and isoleucine), which are moderately prone to oxidation, were corrected with a factor 1.06 (Rudemo et al., 1980).

*2.3 Calculations and statistical analyses*

Duodenal and ileal DM flow were calculated based on average marker concentration in relation to daily supply. The daily supply of titanium oxide was adjusted with background titanium oxide in silages (Johansen et al., 2017). Concentration of purines in the microbial pellet and in duodenal chyme was used to estimate duodenal microbial DM flow. Flows were related to DM intake (DMI) and given as g/kg DMI to facilitate comparison of forages.

Statistical analyses were conducted using R 3.3.0 (R Core Team, 2016). A linear regression model including a regression coefficient for silage DM concentration, a fixed effect of farm (farm 1 or 2), a fixed effect of cut number (spring growth or first regrowth), and an interaction between farm and cut number were used to evaluate differences in chemical composition between silages (n = 8). An adjusted variance test was used to test significance level.

To evaluate the linear effect of silage DM concentration on the various animal measurements, a random regression model including a fixed regression coefficient for silage DM concentration, a random effect of cow (n = 4), a random effect of cut number within farm, and a random regression coefficient for silage DM concentration within cut number for each farm were used. Due to a dubious sample and outlier results, ileal CP and AA flow for one cow in one period were discarded in the analysis. The predicted value at average silage DM concentration (448 g/kg) and the predicted response for a given dependent variable, when increasing silage DM concentration with

100 g/kg, are presented in tables. Parametric bootstrapping with 10,000 repetitions was used to test the effect of silage DM concentration. P-values ≤ 0.05 were regarded as significant, whereas P-values ≤ 0.10 were regarded as tendencies. Further details on statistical analyses are given by Johansen et al. (2017).

## 3. Results

### *3.1. Silages and intake*

The chemical composition of silages is presented in details by Johansen et al. (2017). Silage DM concentration covered the range from 283 to 725 g/kg (Table 1) and did not affect ash, aNDFom and CP concentrations in DM. Proportion of AA-N out of total N increased, whereas proportions of soluble N and $NH_3$-N out of total N decreased with increased silage DM concentration. Total AA concentration in DM was not affected by silage DM concentration. When increasing silage DM concentration, silage AA profile changed; the proportion of arginine increased and the proportions of cysteine and proline tended to increase, whereas the proportions of alanine, histidine, isoleucine, lysine, methionine, ornithine, serine, threonine and valine decreased and the proportions of glycine and leucine tended to decrease. Proportions of aspartate, glutamate and phenylalanine were unaffected by silage DM concentration. Glutamate and histidine proportions were higher in first regrowth silages than in spring growth silages; whereas aspartate and serine proportions were higher in silages from farm two compared to silages from farm one.

The cows consumed on average 12.4 kg DM/day and 1.42 kg AA/day (Table 2), and these intakes were not affected by silage DM concentration. Intake of proline increased by 21.9 g/day, when increasing silage DM concentration with 100 g/kg, whereas daily intake of the remaining AA was not significantly affected.

169 *3.2. Duodenal flow*

170 Total duodenal AA flow per kg DMI tended to increase (P = 0.079, Table 3) with increased silage
171 DM concentration. Proportion of histidine in total duodenal AA flow decreased, lysine proportion
172 in total duodenal AA flow tended to decrease and glutamate proportion in total duodenal AA flow
173 tended to increase with increased silage DM concentration (Table 3). When partitioning total
174 duodenal AA flow per kg DMI into duodenal microbial AA flow per kg DMI and duodenal
175 feed/endogenous AA flow per kg DMI both tended to increase (P = 0.059 and P = 0.070,
176 respectively) with increased silage DM concentration. In microbial AA profile, serine proportion
177 increased and valine proportion decreased, whereas aspartate proportion tended to increase and
178 ornithine proportion tended to decrease. Overall, microbial composition (Table 4) was not affected
179 by silage DM concentration. In feed/endogenous AA profile, cysteine and histidine proportions
180 decreased, lysine proportion tended to decrease and glutamate proportion tended to increase.
181 Glycine constituted 16.8 g/100 g duodenal feed/endogenous AA and had a high standard error in the
182 response to changes in silage DM concentration compared to the other AA.
183
184 *3.3. Digestibility and digestion*

185 Apparent small intestinal AA digestibility increased by 0.013 (P = 0.034) when increasing silage
186 DM concentration with 100 g/kg (Table 5). Digestibility of each individual AA increased with
187 increased silage DM concentration as well, however only as a tendency for alanine, aspartate,
188 lysine, ornithine and serine, and only numerically for cysteine and glycine. The increase in small
189 intestinal AA digestibility together with the tendency towards an increased duodenal AA flow
190 increased the amount of total AA digested in the small intestine by 5.59 g/kg DMI (P = 0.024) when
191 increasing silage DM concentration with 100 g/kg (Table 6). The amount digested per kg DMI
192 increased for all individual AA except ornithine, however only as a tendency for cysteine, histidine

193 and phenylalanine and only numerically for glycine. When increasing silage DM concentration,
194 minor effects were observed in digested AA profile; the proportion of lysine decreased by 0.10
195 g/100 g digested AA (P = 0.049), the proportion of histidine tended to decrease by 0.03 g/100 g
196 digested AA (P = 0.063) and the proportion of glutamate tended to increase by 0.07 g/100 g
197 digested AA (P =0.061) when increasing silage DM concentration with 100 g/kg.
198
199 **4. Discussion**
200 *4.1. Silage AA profile*
201 The increase in silage AA-N as proportion of total N with increased silage DM concentration was
202 most probably caused by reduced proteolysis with higher DM concentration (Slottner and
203 Bertilsson, 2006). Silage AA profile was affected by silage DM concentration. The most
204 pronounced effect was in proline proportion, which increased and thereby increased daily proline
205 intake significantly when increasing silage DM concentration. The increase in proline proportion is
206 consistent with the fact that proline is synthesised during wilting (Kemble and MacPherson, 1954).
207 Proline synthesis occurs as a response to the osmotic stress the plant material is exposed to during
208 wilting, as proline acts as an osmolyte (Delauney and Verma, 1993). The increase in silage arginine
209 proportion when increasing DM concentration has previously been related to lactic acid bacteria
210 that mainly deaminate arginine (Ohshima and McDonald, 1978). In the current silages, a higher DM
211 concentration is associated with a lower concentration of lactate (Johansen et al., 2017), which
212 shows that fermentation by lactic acid bacteria is reduced, and therefore, lactic acid bacteria do
213 probably not deaminate arginine to same extent, as in the silages with lower DM concentration. The
214 higher proportions of arginine and proline, and the general decrease in proportions of nearly all
215 other AA in silages wilted to a higher DM concentration is in accordance with Edmunds et al.
216 (2014).

*4.2. Duodenal AA flow*

Total duodenal AA flow per kg DMI increased with increased silage DM concentration, and this increase was caused by both a higher microbial AA synthesis and a higher AA flow from feed/endogenous sources. The increase in AA flow from feed/endogenous sources was most probably caused by an increase in AA from RUP, as reported by Johansen et al. (2017). However, no marked effect of silage DM concentration on duodenal feed/endogenous AA profile was observed despite the pronounced effect of silage DM concentration on silage AA profile. Even though the ensiling process contributes with 49 % of the variability in AA profile between forages (Givens and Rulquin, 2004), González et al. (2009) have shown, that the AA profile of RUP is similar for green and ensiled Italian ryegrass, although the initial AA profile for the two differed. Similarly, Edmunds et al. (2013) reported a more similar AA profile of RUP between forages, than between RUP AA profile and original AA profile within forage. Thereby, changes in silage AA profile will not affect AA profile of RUP significantly. However, differences in rumen degradation between different feed AA affected by conservation (González et al., 2009) can probably explain the minor effects of silage DM concentration on duodenal feed/endogenous AA profile, where proportions of cysteine and histidine decreased with increased silage DM concentration. Although proline accounted for a large proportion in silage AA profile (7.27-16.0 g/100 g AA), the proline proportion decreased to average 4.42 g/100 g AA in duodenal feed/endogenous AA flow, which might be related to the fact that a larger part of proline compared to other AA is water-soluble (Skiba et al., 1996; González et al., 2009) and thereby more easily degraded in the rumen. The proportion of glycine increased from average 6.05 g/100 g AA in silages to average 16.8 g/100 g AA in duodenal feed/endogenous AA flow. This substantial increase was most probably related to the fact that glycine is the main AA in bile (Larsen et al., 2000) and some bile will end up in the

241 duodenal sample. The large standard error for glycine proportion in duodenal feed/endogenous AA
242 flow, total duodenal AA flow and the amount of AA digested in the small intestine indicated that
243 glycine proportion was affected more by other sources, such as endogenous bile supply, than by
244 silage DM concentration, by which any potential effect of silage DM concentration on glycine
245 proportion was masked.
246 Overall, the reported microbial AA profile corresponded to microbial AA profiles reported in other
247 studies (Larsen et al., 2000; Korhonen et al., 2002; Sok et al. 2017). Silage DM concentration did
248 not affect microbial composition and had only minor effects on microbial AA profile. The exact
249 reason for the small changes in microbial AA profile is unclear but could be related to changes in
250 microbial community caused by changed nutrient supply to the microbes e.g. sugar (Johansen et al.,
251 2017) and rumen degraded AA as previously discussed.
252 The minor changes in microbial AA profile and duodenal feed/endogenous AA profile
253 counterbalanced partly, whereby only a minor decrease in histidine proportion, a tendency towards
254 a minor decrease in lysine proportion and a tendency towards a minor increase in glutamate
255 proportion were found in total duodenal AA profile when increasing silage DM concentrations, all
256 driven by changes in duodenal feed/endogenous AA profile.
257
258 *4.3. Digestion and digestibility*
259 The apparent small intestinal digestibility of individual AA increased with increased silage DM
260 concentration, which is in accordance with the fact that potential digestible AA that passes un-
261 degraded through the rumen has a high digestibility (Skórko-Sajko et al., 1994). Despite not
262 significant, Merchen and Satter (1983) also reported an increased small intestinal digestibility for all
263 AA when cows were fed alfalfa silage with 660 g DM/kg compared to 400 g DM/kg. The lower
264 apparent small intestinal digestibility for cysteine compared to the other AA is in accordance with

values presented by Skórko-Sajko et al. (1994). Small intestinal digestibility of feed glycine do not differ from that of other AA (Skórko-Sajko et al., 1994), whereby the current relatively high apparent small intestinal digestibility of glycine compared to the other AA may be related to the assumption that a high proportion of glycine derived from bile, and endogenous glycine has a high re-absorption (approx. 89 %) in the small intestine (Larsen et al., 2001).

For all AA, the amount digested per kg DMI increased with increased silage DM concentration, though not significantly for all AA. The reduced lysine proportion of digested AA when increasing silage DM concentration might be related to the fact that lysine was one of the AA, where the proportion was reduced most in the silages, when increasing silage DM concentration. As discussed, this was also reflected in a tendency towards a minor decrease for lysine proportion in total duodenal AA flow when increasing silage DM concentration, and simultaneous, silage DM concentration did not increase small intestinal lysine digestibility significantly. Further, histidine proportion of digested AA tended to decrease, probably because of the lower histidine proportion in total duodenal AA flow. Lysine, methionine and histidine are normally considered as the first limiting AA for milk production in dairy cows (Fraser et al., 1991; Kim et al., 1999; 2000). In grass-based diets, histidine proportion is low and histidine is normally the first limiting AA (Vanhatalo et al., 1999; Korhonen et al., 2000; Huhtanen et al., 2002), whereby pre-wilting to a higher DM concentration will aggravate this effect. Therefore, a lower production response than expected based merely on total MP supply will probably appear when increasing silage DM concentration, if lysine or histidine limit milk production.

## 5. Conclusion

Small intestinal digestibility of individual AA and the amount of individual AA digested per kg DMI increased in lactating dairy cows when increasing DM concentration in grass-clover silage by

289 extending pre-wilting. Although the absolute amount digested of all individual AA increased with
290 increased silage DM concentration, the AA profile of digested AA changed by reducing lysine and
291 histidine proportions and by increasing glutamate proportion. Based only on total MP supply, a
292 lower production response than expected will probably appear when increasing silage DM
293 concentration, as either histidine or lysine often are the first limiting AA.
294
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396 Table 1. Chemical composition of experimental grass-clover silages.

| | Farm 1 | | | | Farm 2 | | | | Δ by 100 g/kg | | P-values | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Spring growth | | First regrowth | | Spring growth | | First regrowth | | DM increase | SE[1] | DM | Farm | Cut No. | Farm*Cut No |
| DM (g/kg fresh matter) | 283 | 644 | 322 | 427 | 492 | 660 | 377 | 725 | | | | | | |
| Ash (g/kg DM) | 84.0 | 85.1 | 126 | 139 | 89.0 | 81.9 | 120 | 88.8 | -3.53 | 3.15 | 0.343 | 0.532 | 0.037 | 0.216 |
| aNDFom (g/kg DM) | 429 | 484 | 390 | 390 | 490 | 480 | 364 | 381 | +8.25 | 3.99 | 0.131 | 0.795 | 0.006 | 0.102 |
| CP (g/kg DM) | 158 | 149 | 176 | 180 | 133 | 125 | 148 | 163 | +0.41 | 2.14 | 0.861 | 0.013 | 0.007 | 0.879 |
| N (g/100 g total N) | | | | | | | | | | | | | | |
| Soluble N | 63.2 | 42.4 | 56.1 | 52.2 | 59.2 | 39.8 | 59.1 | 29.5 | -7.40 | 1.14 | 0.007 | 0.248 | 0.089 | 0.796 |
| $NH_3$-N | 5.06 | 1.94 | 5.97 | 5.83 | 3.47 | 2.04 | 6.09 | 1.43 | -1.03 | 0.17 | 0.009 | 0.883 | 0.068 | 0.482 |
| AA-N | 60.1 | 63.3 | 62.2 | 63.6 | 61.7 | 64.4 | 62.6 | 65.1 | +0.90 | 0.15 | 0.010 | 0.897 | 0.025 | 0.328 |
| Total AA (g/kg DM) | 113 | 111 | 131 | 135 | 94.7 | 94.5 | 109 | 124 | +1.61 | 1.37 | 0.325 | 0.006 | 0.002 | 0.974 |
| Amino acids (g/100 g AA) | | | | | | | | | | | | | | |
| Alanine | 8.80 | 7.41 | 8.66 | 7.90 | 8.35 | 7.72 | 8.03 | 7.31 | -0.32 | 0.07 | 0.017 | 0.530 | 0.192 | 0.418 |
| Arginine | 2.45 | 5.35 | 3.18 | 3.84 | 5.83 | 5.69 | 3.42 | 5.93 | +0.68 | 0.15 | 0.019 | 0.317 | 0.463 | 0.248 |
| Aspartate | 10.4 | 10.1 | 9.74 | 10.6 | 11.0 | 11.2 | 11.0 | 11.2 | +0.03 | 0.10 | 0.764 | 0.028 | 0.939 | 0.904 |
| Cysteine | 0.86 | 0.94 | 0.96 | 0.92 | 0.96 | 0.98 | 0.90 | 0.98 | +0.02 | 0.01 | 0.084 | 0.673 | 0.559 | 0.131 |
| Glutamate | 10.0 | 10.2 | 11.3 | 10.9 | 9.57 | 10.6 | 10.5 | 11.2 | +0.14 | 0.11 | 0.303 | 0.336 | 0.028 | 0.676 |
| Glycine | 6.32 | 5.61 | 6.20 | 6.11 | 5.97 | 5.88 | 6.24 | 6.05 | -0.12 | 0.04 | 0.060 | 0.270 | 0.241 | 0.663 |
| Histidine | 2.25 | 1.61 | 2.23 | 2.15 | 1.78 | 1.50 | 2.29 | 1.89 | -0.15 | 0.02 | 0.004 | 0.900 | 0.032 | 0.074 |
| Isoleucine | 5.99 | 5.16 | 5.73 | 5.68 | 5.81 | 5.29 | 5.80 | 5.47 | -0.17 | 0.05 | 0.031 | 0.171 | 0.928 | 0.807 |
| Leucine | 9.97 | 8.51 | 9.46 | 9.36 | 9.54 | 8.93 | 9.68 | 9.36 | -0.26 | 0.09 | 0.061 | 0.165 | 0.734 | 0.599 |
| Lysine | 6.32 | 5.07 | 5.64 | 5.95 | 6.21 | 5.33 | 6.45 | 5.54 | -0.30 | 0.08 | 0.033 | 0.072 | 0.970 | 0.517 |
| Methionine | 2.00 | 1.68 | 1.95 | 1.90 | 1.82 | 1.75 | 1.96 | 1.75 | -0.07 | 0.01 | 0.006 | 0.261 | 0.166 | 0.659 |
| Ornithine | 2.43 | 0.00 | 1.39 | 1.41 | 0.17 | 0.00 | 1.54 | 0.00 | -0.49 | 0.12 | 0.024 | 0.643 | 0.663 | 0.289 |
| Phenylalanine | 6.49 | 5.74 | 6.29 | 6.26 | 6.12 | 5.95 | 6.36 | 6.27 | -0.12 | 0.05 | 0.105 | 0.378 | 0.287 | 0.533 |
| Proline | 7.27 | 16.0 | 9.14 | 9.15 | 8.57 | 11.8 | 7.31 | 9.52 | +1.54 | 0.52 | 0.058 | 0.072 | 0.364 | 0.936 |
| Serine | 5.03 | 4.77 | 5.07 | 5.13 | 5.34 | 5.10 | 5.37 | 5.18 | -0.07 | 0.02 | 0.042 | 0.004 | 0.190 | 0.432 |
| Threonine | 5.49 | 4.86 | 5.43 | 5.28 | 5.28 | 5.09 | 5.47 | 5.23 | -0.12 | 0.03 | 0.021 | 0.079 | 0.212 | 0.693 |
| Valine | 7.92 | 6.94 | 7.62 | 7.51 | 7.68 | 7.19 | 7.60 | 7.18 | -0.21 | 0.04 | 0.019 | 0.151 | 0.539 | 0.876 |

397 [1] Standard error for the predicted response for each increase in silage DM concentration of 100 g/kg.

Table 2. Intake of dry matter (DM), total amino acids (AA) and individual AA predicted at average silage DM concentration (488 g/kg) and the predicted response when increasing silage DM concentration with 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[1] | P-value |
|---|---|---|---|---|
| DM intake (kg/day) | 12.4 | +0.26 | 0.39 | 0.588 |
| Total AA intake (kg/day) | 1.42 | +0.06 | 0.06 | 0.441 |
| AA intake (g/day) | | | | |
| Alanine | 112 | -1.96 | 5.44 | 0.749 |
| Arginine | 63.5 | +10.5 | 4.35 | 0.106 |
| Aspartate | 155 | +9.74 | 6.77 | 0.268 |
| Cysteine | 13.1 | +0.58 | 0.63 | 0.471 |
| Glutamate | 147 | +7.14 | 6.97 | 0.418 |
| Glycine | 86.1 | +1.99 | 3.76 | 0.665 |
| Histidine | 28.6 | -0.73 | 1.30 | 0.621 |
| Isoleucine | 80.6 | +0.85 | 3.57 | 0.852 |
| Leucine | 129 | +1.01 | 6.18 | 0.792 |
| Lysine | 85.7 | +0.74 | 4.08 | 0.898 |
| Methionine | 26.4 | +0.15 | 1.08 | 0.917 |
| Ornithine | 13.9 | -4.54 | 2.40 | 0.188 |
| Phenylalanine | 88.4 | +2.20 | 4.00 | 0.653 |
| Proline | 136 | +21.9 | 5.68 | 0.045 |
| Serine | 73.6 | +2.40 | 2.98 | 0.513 |
| Threonine | 74.9 | +1.21 | 3.27 | 0.760 |
| Valine | 106 | +1.34 | 4.58 | 0.813 |

[1] Standard error for the predicted response for each increase in silage DM concentration of 100 g/kg.

402 Table 3. Duodenal amino acid (AA) flow predicted at average silage dry matter (DM) concentration

403 (488 g/kg) and the predicted response when increasing silage DM concentration with 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[1] | P-value |
|---|---|---|---|---|
| Total duodenal AA flow (g/kg DMI) | 145 | +5.63 | 1.98 | 0.079 |
| Duodenal microbial AA flow (g/kg DMI) | 49.9 | +2.12 | 0.77 | 0.059 |
| Duodenal feed/endogenous AA flow (g/kg DMI) | 95.2 | +4.29 | 1.60 | 0.070 |
| Total duodenal AA (g/100g AA) | | | | |
| Alanine | 6.71 | -0.01 | 0.03 | 0.691 |
| Arginine | 4.75 | +0.03 | 0.03 | 0.410 |
| Aspartate | 11.0 | +0.02 | 0.02 | 0.386 |
| Cysteine | 1.71 | -0.03 | 0.02 | 0.175 |
| Glutamate | 12.0 | +0.06 | 0.02 | 0.081 |
| Glycine | 13.0 | -0.02 | 0.20 | 0.882 |
| Histidine | 2.02 | -0.04 | 0.01 | 0.028 |
| Isoleucine | 5.60 | +0.00 | 0.01 | 0.984 |
| Leucine | 8.06 | +0.02 | 0.03 | 0.539 |
| Lysine | 6.83 | -0.04 | 0.02 | 0.053 |
| Methionine | 1.79 | -0.01 | 0.01 | 0.149 |
| Ornithine | 0.15 | +0.00 | 0.00 | 0.141 |
| Phenylalanine | 5.10 | +0.02 | 0.02 | 0.432 |
| Proline | 4.15 | +0.01 | 0.02 | 0.707 |
| Serine | 5.28 | -0.02 | 0.04 | 0.620 |
| Threonine | 5.37 | +0.00 | 0.02 | 0.930 |
| Valine | 6.41 | -0.01 | 0.02 | 0.469 |
| Duodenal microbial AA (g/100 g AA) | | | | |
| Alanine | 8.29 | -0.04 | 0.02 | 0.140 |
| Arginine | 4.92 | 0.00 | 0.01 | 0.899 |
| Aspartate | 13.0 | +0.05 | 0.01 | 0.054 |
| Cysteine | 1.26 | 0.00 | 0.01 | 0.479 |
| Glutamate | 13.7 | +0.01 | 0.02 | 0.663 |
| Glycine | 5.94 | +0.01 | 0.00 | 0.171 |
| Histidine | 1.78 | -0.01 | 0.01 | 0.207 |
| Isoleucine | 6.32 | -0.01 | 0.01 | 0.509 |
| Leucine | 7.88 | 0.00 | 0.01 | 0.979 |
| Lysine | 8.02 | -0.01 | 0.02 | 0.687 |
| Methionine | 2.38 | -0.01 | 0.01 | 0.157 |
| Ornithine | 0.18 | -0.01 | 0.00 | 0.074 |
| Phenylalanine | 4.95 | 0.00 | 0.01 | 0.992 |
| Proline | 3.67 | 0.00 | 0.01 | 0.680 |
| Serine | 4.78 | +0.03 | 0.01 | 0.014 |
| Threonine | 6.00 | +0.03 | 0.01 | 0.126 |
| Valine | 6.89 | -0.03 | 0.01 | 0.029 |
| Duodenal feed/endogenous AA (g/100 g AA) | | | | |

| | | | | |
|---|---|---|---|---|
| Alanine | 5.85 | +0.02 | 0.05 | 0.685 |
| Arginine | 4.66 | +0.05 | 0.05 | 0.413 |
| Aspartate | 10.0 | +0.03 | 0.05 | 0.594 |
| Cysteine | 1.94 | -0.05 | 0.02 | 0.048 |
| Glutamate | 11.1 | +0.07 | 0.03 | 0.091 |
| Glycine | 16.8 | -0.01 | 0.38 | 0.968 |
| Histidine | 2.14 | -0.06 | 0.01 | 0.027 |
| Isoleucine | 5.21 | 0.00 | 0.02 | 0.833 |
| Leucine | 8.15 | +0.04 | 0.06 | 0.530 |
| Lysine | 6.21 | -0.05 | 0.02 | 0.052 |
| Methionine | 1.47 | 0.00 | 0.01 | 0.693 |
| Ornithine | 0.13 | 0.00 | 0.00 | 0.401 |
| Phenylalanine | 5.17 | +0.03 | 0.03 | 0.412 |
| Proline | 4.42 | +0.03 | 0.03 | 0.431 |
| Serine | 5.53 | -0.04 | 0.05 | 0.578 |
| Threonine | 5.04 | 0.00 | 0.04 | 0.929 |
| Valine | 6.14 | 0.00 | 0.03 | 0.955 |

404 [1] Standard error for the predicted response for each increase in silage DM concentration of 100 g/kg.

405 Table 4. Composition of microbes isolated from the rumen predicted at average silage dry matter
406 (DM) concentration (488 g/kg) and the predicted response when increasing silage DM
407 concentration with 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[1] | P-value |
|---|---|---|---|---|
| OM (g/kg microbial DM) | 715 | -6.41 | 5.05 | 0.263 |
| CP (g/kg microbial DM) | 461 | -3.75 | 4.56 | 0.462 |
| Total AA (g/kg microbial DM) | 352 | -5.54 | 4.27 | 0.277 |
| AA-N (g/100 g microbial N) | 65.0 | -0.27 | 0.37 | 0.657 |
| Total AA (g/16 g microbial N) | 76.3 | -0.27 | 0.43 | 0.730 |
| Purines (g/16 g microbial N) | 19.2 | -0.35 | 0.19 | 0.153 |

408 [1] Standard error for the predicted response for each increase in silage DM concentration of 100 g/kg.

409 Table 5. Apparent small intestinal digestibility of total amino acids (AA) and individual AA
410 predicted at average silage dry matter (DM) concentration (488 g/kg) and the predicted response
411 when increasing silage DM concentration with 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[1] | P-value |
|---|---|---|---|---|
| Total AA | 0.686 | +0.013 | 0.004 | 0.034 |
| Alanine | 0.623 | +0.014 | 0.005 | 0.051 |
| Arginine | 0.732 | +0.021 | 0.004 | 0.011 |
| Aspartate | 0.677 | +0.010 | 0.004 | 0.062 |
| Cysteine | 0.554 | +0.006 | 0.007 | 0.411 |
| Glutamate | 0.642 | +0.014 | 0.004 | 0.033 |
| Glycine | 0.835 | +0.005 | 0.004 | 0.362 |
| Histidine | 0.651 | +0.016 | 0.004 | 0.019 |
| Isoleucine | 0.673 | +0.015 | 0.004 | 0.030 |
| Leucine | 0.678 | +0.019 | 0.004 | 0.016 |
| Lysine | 0.730 | +0.009 | 0.004 | 0.097 |
| Methionine | 0.640 | +0.022 | 0.005 | 0.031 |
| Ornithine | 0.380 | +0.015 | 0.008 | 0.062 |
| Phenylalanine | 0.674 | +0.022 | 0.004 | 0.014 |
| Proline | 0.605 | +0.013 | 0.005 | 0.040 |
| Serine | 0.664 | +0.010 | 0.005 | 0.055 |
| Threonine | 0.647 | +0.011 | 0.005 | 0.046 |
| Valine | 0.663 | +0.013 | 0.004 | 0.034 |

412 [1] Standard error for the predicted response for each increase in silage DM concentration of 100 g/kg.

413 Table 6. Amount of total amino acid (AA) and individual AA digested in the small intestine and
414 composition of digested AA predicted at average silage dry matter (DM) concentration (488 g/kg)
415 and the predicted response when increasing silage DM concentration with 100 g/kg.

| | Predicted at average DM concentration | Δ by 100 g/kg DM increase | SE[1] | P-value |
|---|---|---|---|---|
| Amount digested in small intestine (g/kg DMI) | | | | |
| Total AA | 99.4 | +5.59 | 1.45 | 0.024 |
| Alanine | 6.06 | +0.35 | 0.10 | 0.030 |
| Arginine | 5.00 | +0.34 | 0.10 | 0.045 |
| Aspartate | 10.9 | +0.64 | 0.16 | 0.012 |
| Cysteine | 1.39 | +0.06 | 0.04 | 0.094 |
| Glutamate | 11.2 | +0.71 | 0.17 | 0.019 |
| Glycine | 15.7 | +0.62 | 0.45 | 0.312 |
| Histidine | 1.89 | +0.07 | 0.03 | 0.071 |
| Isoleucine | 5.47 | +0.33 | 0.08 | 0.019 |
| Leucine | 7.91 | +0.50 | 0.14 | 0.032 |
| Lysine | 7.21 | +0.34 | 0.10 | 0.032 |
| Methionine | 1.65 | +0.10 | 0.03 | 0.043 |
| Ornithine | 0.08 | 0.00 | 0.00 | 0.202 |
| Phenylalanine | 4.93 | +0.31 | 0.11 | 0.076 |
| Proline | 3.65 | +0.22 | 0.06 | 0.015 |
| Serine | 5.12 | +0.27 | 0.10 | 0.015 |
| Threonine | 5.06 | +0.29 | 0.08 | 0.015 |
| Valine | 6.18 | +0.35 | 0.10 | 0.020 |
| AA digested in small intestine (g/100 g AA) | | | | |
| Alanine | 6.08 | +0.01 | 0.03 | 0.738 |
| Arginine | 5.05 | +0.06 | 0.05 | 0.308 |
| Aspartate | 10.9 | -0.02 | 0.03 | 0.564 |
| Cysteine | 1.39 | -0.03 | 0.03 | 0.470 |
| Glutamate | 11.2 | +0.07 | 0.03 | 0.061 |
| Glycine | 15.9 | -0.17 | 0.27 | 0.482 |
| Histidine | 1.91 | -0.03 | 0.01 | 0.063 |
| Isoleucine | 5.48 | +0.02 | 0.02 | 0.185 |
| Leucine | 7.92 | +0.06 | 0.06 | 0.407 |
| Lysine | 7.30 | -0.10 | 0.03 | 0.049 |
| Methionine | 1.66 | +0.01 | 0.01 | 0.314 |
| Ornithine | 0.08 | +0.00 | 0.00 | 0.930 |
| Phenylalanine | 4.97 | +0.08 | 0.04 | 0.256 |
| Proline | 3.67 | +0.03 | 0.03 | 0.414 |
| Serine | 5.13 | -0.02 | 0.06 | 0.720 |
| Threonine | 5.07 | 0.00 | 0.03 | 0.925 |
| Valine | 6.18 | -0.01 | 0.02 | 0.729 |

416 [1] Standard error for the predicted response for each increase in silage DM concentration of 100 g/kg.

## 5.5 Paper V – Comparison of protein degradation in the rumen measured *in situ* and *in vivo*

Marianne Johansen and Martin R. Weisbjerg

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# Comparison of protein degradation in the rumen measured in situ and in vivo

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## Abstract

The aim of this study was to compare whether the effect of increased dry matter (DM) concentration in grass-clover silage on rumen protein degradation measured *in situ* reflect the actual *in vivo* change in protein degradation in the rumen. Eight grass-clover silages with DM concentrations ranging from 283 to 725 g $kg^{-1}$ were fed *ad libitum* as the sole feed to four rumen and duodenal fistulated Holstein dairy cows in a crossover design. Based on duodenal samples, three external markers and purine concentration in isolated rumen microbes, microbial protein flow at the duodenum and true rumen digestibility of feed protein were estimated. Additionally, the silages were incubated in the rumen (*in situ*) of three cows in Dacron bags with eight incubation times, and the effective protein degradation was calculated assuming a rate of passage of 0.08 $h^{-1}$. The protein degradation was reduced with 36 and 44 g $kg^{-1}$ when increasing the silage DM concentration with 100 g $kg^{-1}$ for *in situ* and *in vivo* measurements, respectively. The slope from regression of *in vivo* on *in situ* was 0.91, but not significantly different from one. In conclusion, *in situ* measurements reflect the *in vivo* change in true rumen protein degradation well.

**Keywords:** protein degradation, *in situ*, *in vivo*, forage

## Introduction

The amount of absorbed protein is a major factor affecting the milk production in dairy cows (Allen, 1996). Therefore it is important to have a high and stable forage production with a high protein quality. Most forage used for milk production is conserved as silage, but the dry matter (DM) concentration in the plant material before ensiling will affect the microbial fermentation during the ensiling process. A higher DM concentration reduces the microbial fermentation, by which the final silage has a higher concentration of sugar and true protein and a lower concentration of fermentation products (Harrison *et al.*, 1994). When feeding this silage, the microbial synthesis in the rumen will increase and a higher proportion of undegraded feed protein will reach the small intestine (Johansen and Weisbjerg, 2015). In feed evaluation systems most parameters are determined by *in situ* or *in vitro* techniques and it is important that these techniques reflect what actually happen *in vivo* when they are used to optimise feed rations. Despite widespread use of the *in situ* technique very few *in situ-in vivo* evaluations have been published. Therefore, the objective of this study was to test whether the reduced rumen protein degradation with increased DM concentration in grass-clover silage measured *in situ* reflect the actual *in vivo* change in protein degradation in the rumen.

## Materials and methods

Spring growth and first regrowth of grass-clover swards grown by two Danish organic farmers were cut and pre-wilted to a planned DM concentration of 350 and 700 g $kg^{-1}$, resulting in total eight silages ensiled without additives and with DM concentrations ranging from 283 to 725 g $kg^{-1}$. For *in vivo* determination of rumen degradation of crude protein (CP) two primiparous and two multiparous Holstein dairy cows (551±33 kg body weight, 216±23 days in milk, mean ± SD) fistulated in rumen and duodenum were fed *ad libitum* with the silages in a crossover design, with 5 periods of 3 weeks. No concentrate was offered, but minerals and vitamins were offered daily. Three markers (10 g $Cr_2O_3$, 10 g $TiO_2$ and 2 g $YbCl_3 \cdot 6H_2O$) were dosed in the rumen twice a day for measurement of flow at duodenum.

In the last week of each period daily feed intake was registered and 12 subsamples from the duodenum were collected over 94 h to cover the diurnal variation, pooled, and subsequently analysed. Once in each period, microbes were isolated from the rumen fluid and analysed for CP and purines to estimate the flow of microbial protein at the duodenum. The flow of nutrients in the duodenum was calculated based on the average of the concentration of each marker in relation to daily supply. True rumen digestibility of CP was calculated as feed intake minus duodenal flow corrected for microbial contribution divided by feed intake. It was assumed that the endogenous flow of CP in the duodenum was constant between treatments and therefore not corrected for. Statistical analyses were done in R (R Core Team, 2014) using a linear random regression model with DM concentration as fixed effect, cow and growth × farmer as random intercepts and with a growth × farmer random slope.

Additionally, the silages were dried, milled (1.5 mm cutter mill) and weighed out in Dacron bags (pore size: 38×38 µm, effective size: 10×7.5 cm) for *in situ* determination of rumen degradation of CP, and incubated in the rumen for 0, 2, 4, 8, 16, 24, 48 and 96 hours, respectively. For each of the silages, one bag was used for each incubation time and repeated in three fistulated dry cows fed at maintenance. After incubation all samples were washed, dried and analysed for the concentration of CP. Using non-linear regression in R (R Core Team, 2014) degradation profile parameters (Ørskov and McDonald, 1979) for each silage were estimated according to the following equation:

$$\text{Degradation profile}(t) = a + b(1 - e^{-ct}),$$

where a is the soluble fraction, b is the insoluble but rumen degradable fraction, c is the fractional rate of degradation of fraction b ($h^{-1}$) and t is incubation time (h). Based on the estimated parameters the effective protein degradation (EPD) was calculated for each of the silages by the following equation (Ørskov and McDonald, 1979):

$$EPD = a + b(c / c+k),$$

where k is the fractional rate of passage out of the rumen set to 0.08 $h^{-1}$, as no correction for particle loss was made. A linear regression with DM concentration as fixed effect was performed.

The *in vivo* degradability were compared with the calculated EPD and the estimated parameters a, b and c in a linear random regression model with EPD, a, b or c as fixed effect, cow and growth × farmer as random intercepts and with a growth × farmer random slope.

## Results and discussion

The DM intake of the cows (12.5±2.1 kg $d^{-1}$, mean ± SD) was not affected by the DM concentration in the silage ($P$=0.6) in the *in vivo* experiment. It is therefore assumed that the rate of passage out of the rumen was not affected either. The true rumen degradation measured *in vivo* decreased with 44 g $kg^{-1}$ ($P$=0.02) when increasing the DM concentration in the silage with 100 g $kg^{-1}$ (Figure 1). In comparison, the EPD measured *in situ* decreased by 36 g $kg^{-1}$ ($P$<0.001) when increasing the DM concentration in the silage with 100 g $kg^{-1}$. The measured EPD values varied from 715 g $kg^{-1}$ in the silage with the highest DM concentration to 884 g $kg^{-1}$ in the silage with lowest DM concentration. As seen in Figure 1, the actual values for the true rumen digestibility measured *in vivo* were considerable smaller than the EPD values. This indicates that the contribution of CP from endogenous sources was high in the duodenal flow, which probably partly was due to the relatively low feed intake.

The regression of the true protein degradation measured *in vivo* on EPD measured *in situ* was significant ($\alpha$=0.91, $P$=0.02) and did not differ from one ($P$=0.8) showing that the change in protein degradation

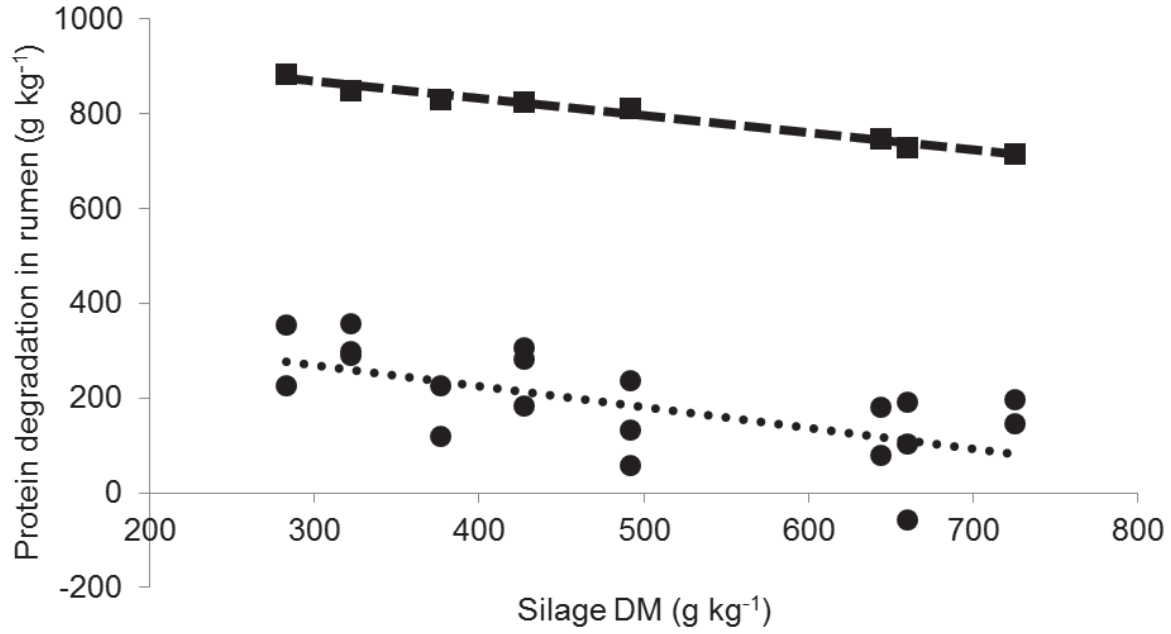


Figure 1. True rumen protein degradation determined *in vivo* (●, n=20) with the regression line (••••, y = −0.44 + 401) and the effective protein degradation determined *in situ* (■) with the regression line (▬ ▬, y = −0.36x + 976).

measured *in vivo* is reflected well in *in situ* measurements. The *in vivo* degradability were positively correlated to the soluble fraction a ($P$=0.02), negatively correlated to the insoluble but degradable fraction b ($P$=0.03) but not correlated to the fractional rate of degradation ($P$=0.6). When correcting the degradation parameters for particle loss, estimated as the difference between zero hour solubility and solubility over filter paper, and using 0.05 $h^{-1}$ passage rate for calculation, the regression coefficient between *in vivo* and *in situ* measurements was close to one, but not significant ($\alpha$=0.97, $P$=0.17).

## Conclusions

It is concluded that the change in rumen protein degradation, when increasing the DM concentration of grass-clover silage measured *in vivo,* is well reflected in the EPD measured *in situ*.

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## 5.6 Paper VI – Leaf:stem ratio as a tool to estimate field losses

Marianne Johansen, Karen Søegaard and Martin R. Weisbjerg

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# Leaf:stem ratio as a tool to estimate field losses

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## Abstract

Silage production from green forages often includes a pre-wilting in the field with a potential loss of plant material. The loss from the field has seldom been determined, but is expected to be high especially for legumes pre-wilted to high dry matter concentration. Under the assumption, that mainly leaf material is lost in the field, the change in leaf:stem ratio through the harvest management could be used as a tool to estimate field losses. To test this idea, the leaf:stem ratio was determined in samples collected before mowing, immediately after mowing, and after raking in fields with primary growth of pure perennial ryegrass (early and late harvested), festulolium, tall fescue and red clover. The estimated field losses were 8.7% and 2.3% in early and late harvested perennial ryegrass, respectively, 6.8% in festulolium, 24% in tall fescue and 12% in red clover. A high positive correlation (0.95) was observed between leaf:stem ratio in the initial sample and the estimated loss. It is concluded that leaf:stem ratio is a potential tool to estimate field losses.

**Keywords:** field loss, pre-wilting, forage, silage production, leaf:stem ratio

## Introduction

In dairy farming it is important to have a high and stable forage production, and most green forages used for feeding are conserved as silage after a pre-wilting period in the field. In Denmark it is recommended to pre-wilt green forages to a dry matter (DM) concentration of approx. 350 g $kg^{-1}$ as the in-silo DM losses due to effluent decrease with increasing DM concentration up to approx. 300 g $kg^{-1}$ where it stop (Zimmer and Wilkins 1984). Concurrently, the pre-wilting period causes losses due to respiration, due to leaching by rain and due to mechanical treatment (McGechan, 1989). Field losses are expected to increase with increasing pre-wilting (Zimmer and Wilkins, 1984), especially for legumes pre-wilted to high DM concentration. Increased pre-wilting will reduce the microbial fermentation under the ensiling process, by which the protein value for dairy cows is increased (Johansen & Weisbjerg, 2015), but if field losses are high the total protein yield is reduced. The losses from the field during mowing, pre-wilting and raking have seldom been determined. At farm level it will be useful to have a tool to estimate field losses, which can be used to improve harvest management and consequently reduce field losses. Under the assumption, that mainly leaf material is lost in the field, the change in leaf:stem ratio in plant material collected in different steps of the harvest management can be used as a tool to estimate the field losses, and the objective of this paper is to test the practicability of this idea.

## Materials and methods

Fields with pure perennial ryegrass (*Lolium perenne* L., cv. Calvano 1), festulolium (*Festulolium braunii* K.A cv. Perun), tall fescue (*Festuca arundinacea* Schreb., cv. Tower) and red clover (*Trifolium pratense* L., cv. Suez) were established in the start of April 2014 with barley (*Hordeum vulgare* L.) as cover crop on AU Foulum, Aarhus University, Tjele, Denmark. The primary growth of tall fescue, festulolium and part of the perennial ryegrass (early perennial ryegrass) were mown on May 21, 2015, and wilted for three days. In the middle of the wilting period it was raining 3.2 mm. The rest of the primary growth of perennial ryegrass (late perennial ryegrass) and the primary growth of red clover were mown on June 3, 2015, and wilted for two days. The goal with the wilting was to achieve a DM concentration of 350-400 g $kg^{-1}$. For

all cuts the stubble height was set to 7 cm. The crops were wilted on broad swaths covering the whole area. After wilting, the swaths were raked before baling.

Five spots (30×30 cm), randomly selected in each field, were cut to 7 cm stubble height with a shears and pooled within species prior to mowing. Further, one sample covering 40 cm length of the swath width of a random selected swath was collected immediately after mowing and after raking in each field. All samples were representative reduced to a size of approx. 200 g and leaves (leaf blade and petiole) and stems (leaf sheath, stem and flower) were separated by hand and dried at 60 °C for 48 h to determine the leaf:stem ratio on DM basis. The field losses of DM were calculated with the assumption, that only leaf material is lost, whereas the stem quantity is constant. The following equation giving the loss in percentages was used:

$$\text{Field loss of DM (\%)} = (\text{ratio}_1 - \text{ratio}_2) / (\text{ratio}_1 + 1) \times 100$$

where $\text{ratio}_1$ is the first achieved (without loss) leaf:stem ratio and $\text{ratio}_2$ is the second achieved (with loss) leaf:stem ratio. No statistics were made, as the objective was to test the practicability of an idea.

## Results and discussion

The leaf:stem ratio varied before mowing from 0.60 in late perennial ryegrass to 4.07 in tall fescue (Table 1). For festulolium the ratio after mowing was higher than the ratio before mowing, and for late perennial ryegrass and tall fescue the ratio after mowing was lower than the ratio after raking, by which negative field losses appeared (Table 2) when estimating the loss in the individual steps. To use the leaf:stem ratio as a tool to estimate field losses it is important, that the sampling procedure is performed well and is representative. The experience from this study indicates that sampling in the broad swath after mowing was difficult, whereas it was easier to get a representative sample from the narrow swath after raking. Therefore, the most probable estimates for the field losses in the grasses were the total losses from before mowing to after raking, which varied from 2.3% in late perennial ryegrass to 24% in tall fescue (Table 2). For red clover, the leaf:stem ratio before mowing was lower than the leaf:stem ratios after

Table 1. Leaf:stem ratio and the dry matter concentration (DM, g $kg^{-1}$) for the different crops before mowing, after mowing and after raking.

| | Before mowing | | After mowing | | After raking | |
|---|---|---|---|---|---|---|
| | Ratio | DM | Ratio | DM | Ratio | DM |
| Early perennial ryegrass | 1.30 | 199 | 1.21 | 211 | 1.10 | 346 |
| Late perennial ryegrass | 0.60 | 202 | 0.52 | 206 | 0.56 | 372 |
| Festulolium | 0.83 | 163 | 1.05 | 169 | 0.71 | 283 |
| Tall fescue | 4.07 | 194 | 2.31 | 200 | 2.86 | 412 |
| Red clover | 0.74 | 124 | 1.15 | 117 | 0.89 | 335 |

Table 2. Estimated field losses of dry matter (%). Suggested most probable estimates of total field loss are marked with an asterisk.

| | Before mowing to after mowing | After mowing to after raking | Before moving to after raking |
|---|---|---|---|
| Early perennial ryegrass | 3.9 | 5.0 | 8.7* |
| Late perennial ryegrass | 4.8 | -2.6 | 2.3* |
| Festulolium | -12 | 17 | 6.8* |
| Tall fescue | 35 | -17 | 24* |
| Red clover | -23 | 12* | -8.3 |

mowing and after raking. This was probably because the stubble height at 7 cm was not achieved during the mowing, since the red clover has lain down in the field before mowing. Therefore, the leaf:stem ratio after mowing and after raking cannot be compared with the leaf:stem ratio before mowing for red clover in this experiment, which was obvious from the estimated negative field losses. This also demonstrates, that the leaf:stem ratio as a tool to estimate field losses only is valid, if the stubble heights are identical for both samples used for the estimation. Because of the discrepancy in stubble height, the best estimate for the field loss in red clover in this experiment was probably the estimate from after mowing to after raking, which was 12% (Table 2).

The estimated field losses were highly correlated (0.95) with the leaf:stem ratio in the initial sample, indicating that more leaves enhance the risk for field losses. This does also support the assumption, that mainly leaf material is lost in the field, as the estimated total losses will be even higher if stem material is lost as well. As the forage parts dries they become more susceptible to be lost in the field (McGechan, 1989), therefore the final DM concentration in the forage is expected to highly influence the loss. The achieved DM concentrations in the forages were positively correlated (0.57) with the estimated loss, but not in same extent as leaf:stem ratio. Leaf material is drying faster than stem material, by which the DM concentration in the leaf part were appreciably higher in the leaf part compared to the stem part after wilting (data not shown), which increases the risk for losing leaf material.

The leaf:stem ratio as a tool to estimate field losses can only be used in fields, where both leaf and stem material is harvested. In the present experiment a field with pure white clover (*Trifolium repens* L.) was established as well, but due to the stoloniferous growth of white clover, only leaves were harvested in the vegetative phase. A high field loss was expected in this field, but the tool could not be used to detect the loss. Alternatively, the tool possible can be used in a modified way using the leaf blade:petiole ratio, but this idea has not been tested. Contrarily, the tool can be used in mixed grass-white clover fields, as the grass contributes with a stem part.

## Conclusions

Leaf:stem ratio is a potential tool to estimate field losses for forages that have both a stem part and a leaf part. The tool requires representative sampling and a constant stubble height in the samples used for the estimation.

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# 6 General discussion

The overall aims of this PhD project were to obtain knowledge on how green forages affect feed intake and milk production in dairy cows and to obtain knowledge on how pre-wilting of grass-clover before ensiling affects the protein value of the forage. The obtained results are discussed in detail in the included papers (Chapter 5). In this section, reliability and applicability of the results will be discussed in a broader context.

## 6.1 Reliability of meta-analyses

Quality and reliability of a meta-analysis depends on the included studies. Even though the statistical method reduces the subjective assessment of single experiments compared to a review, publication biases can still occur. Sometimes, papers are rejected by journals or studies are not tried published at all because of lack of significant results. According to Moher *et al.* (1999), meta-analyses of clinical randomised controlled trials based solely on published experiments overestimate treatment effects by an average of 12% compared to meta-analyses, which include both published experiments and experiments from "grey" literature that is difficult to locate or retrieve. To overcome this publication bias, all relevant literature in any language, both published and unpublished, must be found and included in the meta-analysis (Phillips, 2005), however, this is not achievable. The publication bias could not be estimated in the current meta-analysis (Paper I), but was considered low, because experiments testing forages in dairy cows are expected to be published regardless of their results, if the experiments are properly conducted. Another important factor for the reliability of a global hypothesis testing meta-analysis is that the intended treatments in the included experiments are not confounded by other factors. In the current meta-analysis, the focus was on different green forage sources, however, numerous experiments were excluded from the meta-analysis because of confounding effects, such as changed forage:concentrate ratios (Broderick *et al.*, 2002; Cherney *et al.*, 2002; Jonker *et al.*, 2002; Cherney *et al.*, 2004), different concentrate compositions (Hansen *et al.*, 1991; Hassanat *et al.*, 2014) or cows being fed concentrate according to milk yield (Randby, 1992; Fisher *et al.*, 1993; Johansen and Nordang, 1994; van Dorland *et al.*, 2008). Excluding these studies from the meta-analysis ensured that differences obtained between different green forage species were caused by the green forage in question and not by other dietary changes.

As discussed in Paper I, only a few studies had compared different pure grass species, which reduced the strength of the estimates and supported that knowledge regarding feed intake and milk production of different grass species is scarce in the literature. However, some publications with confounding effects not included in the meta-analysis have included different grass species, which indicates that the awareness about milk production potential of different grass species may be higher than indicated by the meta-analysis.

In many published studies, mixes of different green forage species were fed, as use of mixes is common practice in dairy farming. These mixes could not be used in the meta-analysis directly because the treatments could not be allocated to a single forage type, which was

necessary in the statistical approach used to analyse Dataset 1, 2 and 3 (see Paper I for specification). However, a regression model taking the proportion of single species in the forages into consideration could deal with the mixes, and this statistical method was used also by Moharrery *et al.* (2014). By using this approach in Dataset 4, compared to the approach in Dataset 3, the number of included treatment means increased from 84 to 161, which increased the reliability of the meta-analysis markedly, as the results were similar.

## 6.2 Response to increased clover proportion

The higher feed intake potential of legume-based diets compared to grass-based diets was confirmed by the meta-analysis (Paper I) and Experiment 1 (Paper II) and resulted in a higher milk production. In practical Danish farming, grass-clover mixtures are normally grown in the fields and used for feeding. Therefore, knowledge on marginal responses to changes in clover proportion will be useful for a combined optimisation of forage and milk production. In the meta-analysis, there was a high level of agreement in predicted responses between Dataset 3 and Dataset 4, which indicated that responses are correlated linearly to the proportion of single species. However, it was not possible to test whether the response was linear or curvilinear. In Experiment 1, increasing the forage clover proportion from 0 to 50% increased DMI and milk yield more than increasing the clover proportion from 50-100%. This resulted in a quadratic effect for white clover, but the quadratic effect was not significant for red clover. As discussed in Paper II, the quadratic effect of increasing the white clover proportion might be caused by physiological regulation of feed intake instead of physical regulation in cows fed pure white clover. However, feed intake and milk production are also affected by forage OM digestibility, and the OM digestibility normally changes

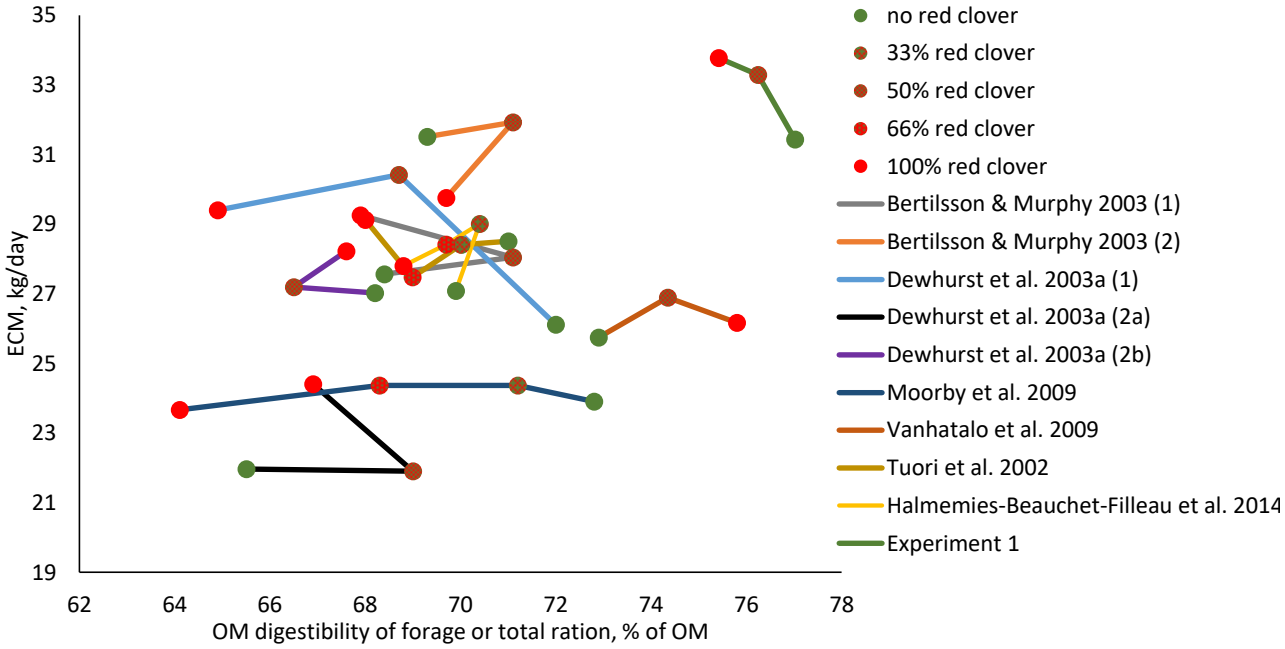


**Figure 6.1.** Relationship between ECM, OM digestibility of forage or total ration and proportion of red clover in forage in previously published experiments and Experiment 1. The colour of the dots represents the proportion of red clover. The number in brackets refers to the experiment number within publication.

when changing the clover proportion, thus there is a confounding effect. Figure 6.1 compares the relationship between ECM yield, OM digestibility of forage or total ration and proportion of red clover in forage in previously published experiments and Experiment 1. The OM digestibility cannot be compared directly among studies, as the method used to determine OM digestibility differed between experiments, but differences within studies should to some extent be comparable. As illustrated in Figure 6.1, Dewhurst *et al.* (2003a)(1) and Moorby *et al.* (2009) both reported a higher ECM production for cows fed mixtures of grass and red clover than for cows fed pure grass or pure red clover. However, in both studies, the OM digestibility decreased markedly by increased proportion of red clover, and therefore it is difficult to separate the effect of OM digestibility and red clover proportion. Based on current knowledge, it is not possible to determine which proportion of legumes that is the most optimal to include in the feed ration, however, inclusion of clover increases feed intake and milk production.

## 6.3 Relationship between ECM yield and OM digestibility

Knowledge on responses of dairy cows to changes in forage OM digestibility is required to optimise the harvesting strategy to produce the highest herbage yield with the most optimal OM digestibility regarding milk production. One of the intended aims with the meta-analysis was to relate DMI and ECM production to OM digestibility to determine the impact of increasing forage OM digestibility. However, this was, as discussed in Paper I, not possible because of lack of variation in OM digestibility within grasses or legumes within experiment in the available data. Furthermore, across the studies included in the meta-analysis, it was not possible to find the most optimal OM digestibility, as the method used to determine OM digestibility differed between experiments, and OM digestibility therefore could not be compared directly among studies, as earlier mentioned. The linear regression of feed efficiency (kg ECM/kg DMI) on OM digestibility presented in Paper I showed that ECM yield increased with 0.1-0.2 kg/day with each percentage point increase in OM digestibility. However, this approach assumed that the response to increased OM digestibility was linear. In Experiment 1 (Paper II), the results suggested that the response in ECM yield can be considered linear when silage OM digestibility is within the range of 73.9-80.6%, meaning that the marginal ECM response is independent of point of origin. The increase in ECM yield equalled 0.6 kg/day for each percentage point increase in silage OM digestibility and was independent of whether the forage consisted solely of grass or included 50% clover. When feeding the grass silage with the highest OM digestibility (83.4%), cows did not produce the expected amount of milk based on the amount of OM actually digested in the gastrointestinal tract, but as discussed in Paper II, no real explanation for this could be found. For the white clover silage, which had an OM digestibility of 82.2%, the intake was probably regulated physiologically instead of physically, as also discussed in Paper II. As mentioned in Paper II, these findings indicated that there is an optimum for silage OM digestibility in relation to ECM production in the range 79-82%, however, the exact optimum could not be determined based solely on data from the meta-analysis or Experiment 1.

Weisbjerg and Johansen (2017) have combined the results from Experiment 1 with previous Danish production experiments performed at AU Foulum in the period from 2004-2016. The included experiments used TMR feeding with a fixed concentrate proportion and composition within experiment and each experiment included at least three levels of forage OM digestibility. The forages included were grass silage, grass-clover silage, and/or maize silage, and the determined OM digestibility was comparable between experiments. The analysis of these studies showed that DMI responded positively up to 85% forage OM digestibility and ECM yield responded positively up to 82% forage OM digestibility, however, the marginal responses decreased with increasing forage OM digestibility (Figure 6.2). Both a quadratic and a logarithmic relationship were tested by Weisbjerg and Johansen (2017), and for both DMI and ECM, the relationship was best described by the logarithmic term. The approach used by Weisbjerg and Johansen (2017) could only test whether the response was linear or curvilinear in the evaluated range of silage OM digestibility, and not, as indicated by the results in Experiment 1, whether the marginal ECM response is constant up to a given optimal OM digestibility. Therefore, the exact optimum for forage OM digestibility regarding milk production is probably within the range 80-82%.

The current knowledge indicates that positive production responses can be achieved by increasing forage OM digestibility up to 80-82%. However, to produce green forages with this level of OM digestibility, the forage must be harvested at an early developmental stage, before pronounced secondary wall thickening and lignification occurs. Simultaneously, this will result in a lower biomass yield in the single cut (Weisbjerg *et al.*, 2010), but will make room for an additional cut during the growing season. Therefore, total biomass yield harvested during the growing season and production costs have to be considered to determine, whether producing green forages with 80-82% OM digestibility is economically profitable. However, economic calculations are beyond the scope of this thesis.

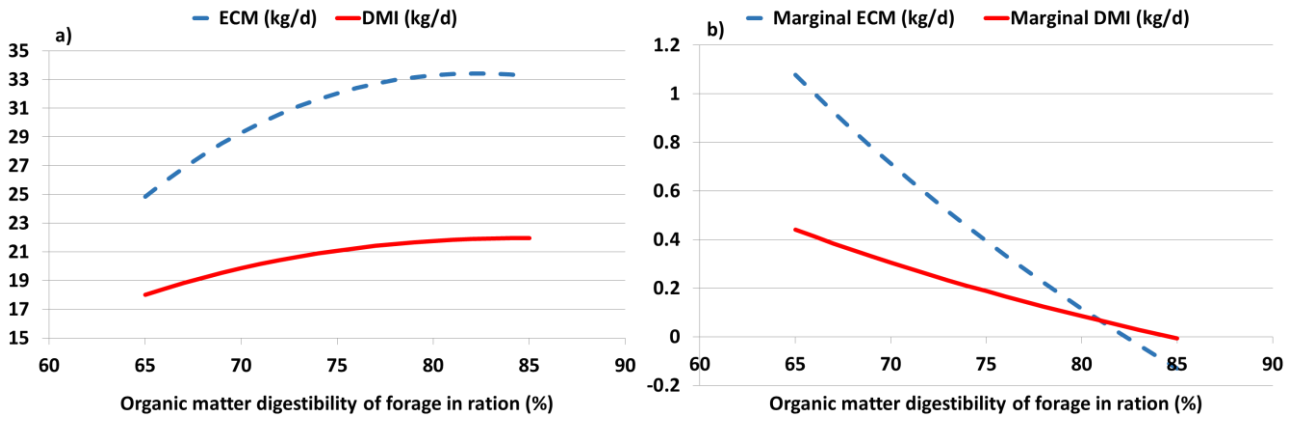


**Figure 6.2.** Estimated response in DMI and yield of ECM (**a**) and estimated marginal response in DMI and yield of ECM (**b**) with increased OM digestibility of forage in ration (Weisbjerg and Johansen, 2017).

## 6.4 Comparison and evaluation of markers

Reliability of results obtained in studies measuring digestion and digestibility of nutrients highly depends on correct estimation of the digesta flow. In Experiment 2 (Paper III and IV), three external flow makers were used. An initial comparison of DM flows calculated based on each single marker showed that using $TiO_2$ as flow marker resulted in deviations in nutrient flow compared to using $Cr_2O_3$ or $YbCl_3 \cdot 6H_2O$. The deviations in nutrient flow were more pronounced in some silages compared to others, and an analysis of $TiO_2$ in the silages revealed, that all silages contained $TiO_2$, and the first regrowth silages had a higher concentration than the spring growth silages (Paper III, Table 1). When correcting the input of $TiO_2$ with the background concentration in the silages, there was a high agreement between duodenal and ileal DM flow and faecal DM output when comparing each marker as shown in Figure 6.3. For all comparisons, the regression line was close to the identity line and the coefficient of determination was generally high ($R^2$=0.84-0.97). As there was a high agreement between the external markers and none of them seems less reliable, an average of the calculated DM flows was used. Besides the external markers, iNDF was analysed as an internal marker and the comparison of iNDF and the external markers is evident in Figure 6.3. A comparison of iNDF and $Cr_2O_3$ showed that the regression line was close to the identity line for duodenal and ileal DM flow, but iNDF predicted a higher faecal DM output than $Cr_2O_3$. The relationship between iNDF and $Cr_2O_3$ in the different digesta samples corresponded to values presented by Lund *et al.* (2007). Generally, the coefficient of determination was lower when comparing iNDF to the other markers ($R^2$=0.53-0.73), than when comparing $Cr_2O_3$, $YbCl_3 \cdot 6H_2O$ and $TiO_2$. Therefore, the flows obtained using iNDF as a marker were not included in the average DM flows used to calculate the flow of other nutrients and digestibility.

The recovery of markers was not determined in the current experiment, but comparing of rumen and total tract NDF digestibility can be used as an indicator of marker dysfunction. If duodenal digesta flow is overestimated, i.e. the recovery of marker is too low, then rumen NDF digestion will be underestimated, and therefore erroneously indicate, that a large proportion of the NDF is digested in the hindgut, provided that faecal output is estimated correctly (Titgemeyer, 1997). In Experiment 2, the average rumen NDF digestibility was 0.761 and average total tract NDF digestibility was 0.770 (Paper III, Table 4). The relationship between these digestibilities corresponded well to the relationship between values presented by Ahvenjärvi *et al.* (2000) where duodenal digesta flow was estimated using the triple-marker method (France and Siddons, 1986) and total tract digestibility was determined by total faecal collection. In the triple marker method, three markers are used and digesta flow is calculated based on marker concentrations in three different phases of the digesta sample, thus separation of the digesta sample is necessary. The advantage of the triple-marker method is that the method can handle non-representative sampling (France and Siddons, 1986). Rumen and total tract NDF digestibility were 0.582 and 0.599, respectively, in the study by Ahvenjärvi *et al.* (2000). These values are in accordance with the fact that less than 5% of the total NDF digestion takes place in the hindgut (section 2.4.1).

If the recovery of a marker is too low in both duodenal and faecal samples, the aforementioned ratio between rumen and total tract NDF digestibility can be obtained, but both digestibilities will be estimated too low. Data on *in situ* rumen NDF degradation for the silages used in Experiment 2 showed that the average effective rumen NDF degradation was 0.612 and 0.785 if passage rates of 2%/h and 0.5%/h, respectively, were used in the calculations (Johansen et al., unpublished data). This indicates that the *in vivo* measured NDF digestibility most probably was not too low, and the recovery of the markers has been sufficient. Because of a relatively low feed intake, a low passage rate was expected, as passage rate is positively correlated to feed intake level (Okine and Mathison, 1991), and this was supported by comparing the *in situ* and *in vivo* rumen NDF degradation.

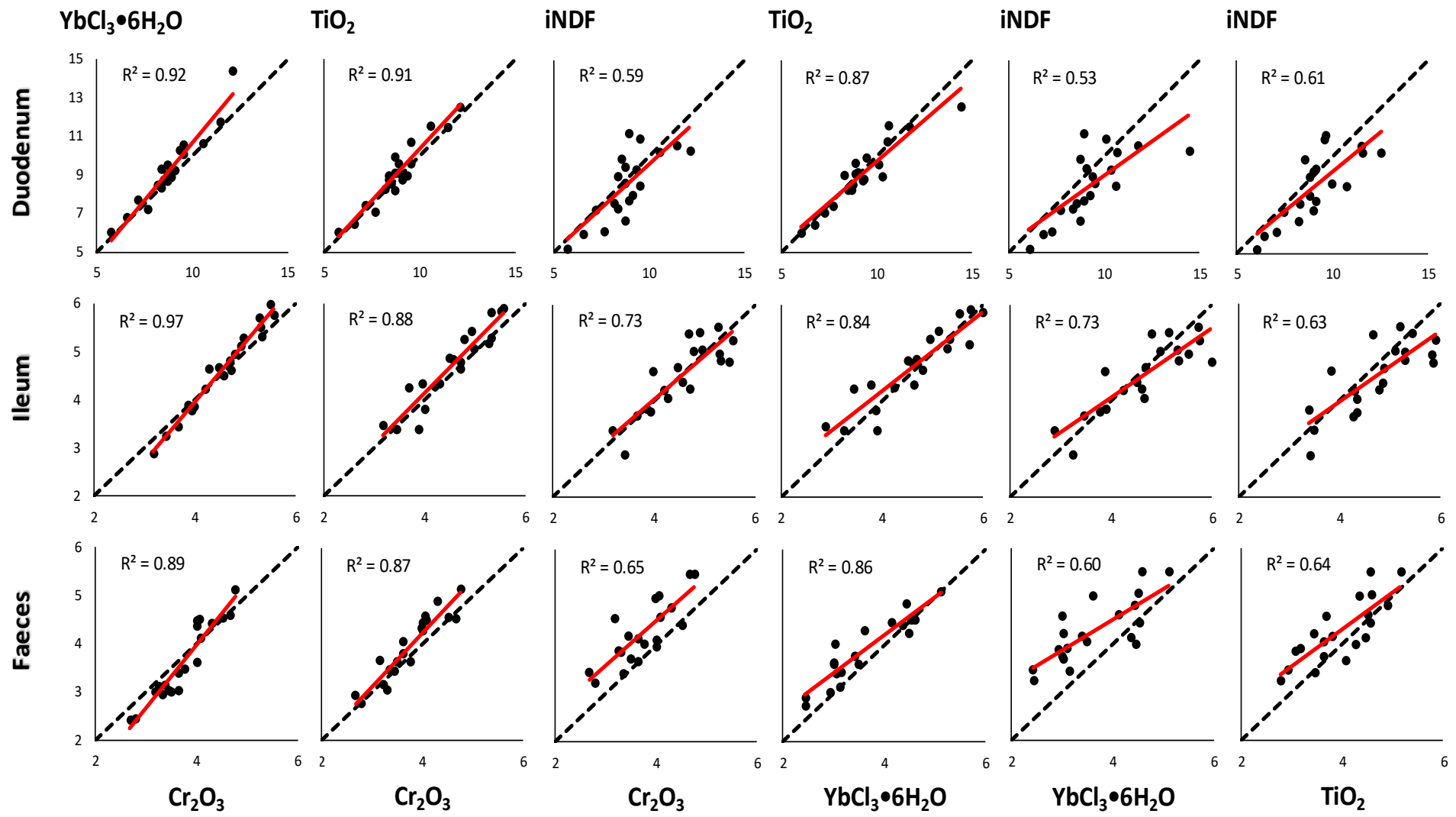


**Figure 6.3.** Comparison of duodenal and ileal DM flow and faecal DM output determined using $Cr_2O_3$, $YbCl_3•6H_2O$, $TiO_2$ or iNDF, respectively, as flow marker in Experiment 2. The dots represent the 20 observations, the red line is the regression line for the actual comparison and the dotted line is the identity line.

## 6.5 Statistical approach in Experiment 2

In Experiment 2 (Paper III and VI), the DM concentration of the two silages within cut were planned to be 350 and 700 g/kg, respectively. This was not achieved for any of the cuts, but the first regrowth silages from Farm 2 were close to the aim with 377 and 725 g DM/kg fresh matter, respectively (Paper III, Table 1). However, for all cuts, the longer pre-wilting increased DM concentration and overall, the DM concentrations covered the range from 283 to 725 g/kg. As the wettest spring growth silage from Farm 2 (492 g DM/kg fresh matter) was drier than the driest first regrowth silage from Farm 1 (427 g DM/kg fresh matter), it was unsuitable to make a variance analysis including DM concentration as a factor with wet and dry as levels, since the levels overlapped (283-492 vs. 427-725 g DM/kg fresh matter). However, the spread of DM concentrations over the range of 283-725 g/kg appeared to be better than having four silages with approximately 350 g DM/kg fresh matter and four silages with approximately 700 g DM/kg fresh matter, as linear regression analysis could then be properly applied. Using the approach with a random regression coefficient for silage DM concentration within cut number for each farm ensured that significance or tendencies for the overall regression line for silage DM concentration were only reached if all cuts responded similarly to changes in DM concentration. Figure 6.4 illustrates the statistical pro-

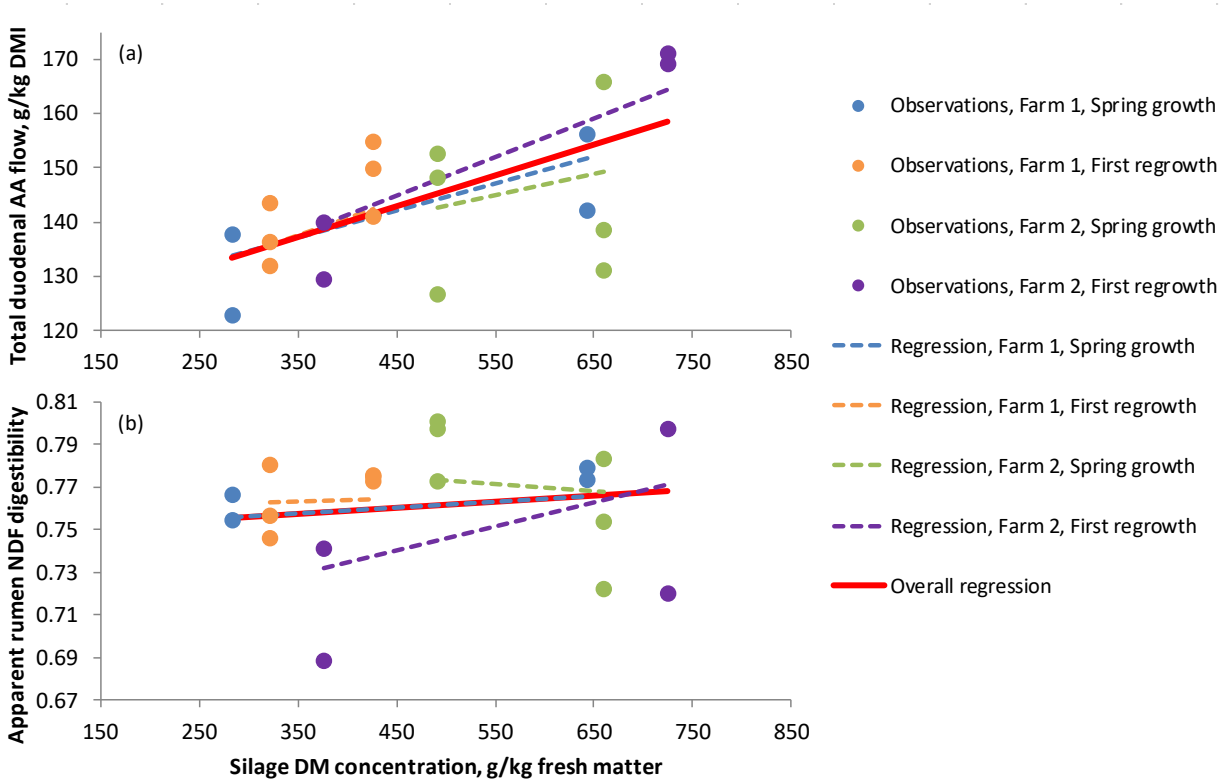


**Figure 6.4.** Total duodenal AA flow (**a**) and apparent rumen NDF digestibility (**b**) as function of silage DM concentration. This illustrates the statistical procedure used in Experiment 2. The dots (•) represent the actual observations. The dotted lines (- - -) represent the random regression lines for each cut within farm when the random effect of cow is taken into account and the line (—) represents the overall fixed regression line taking the random effects into account.

cedure with random regression lines for each cut within farm. In Figure 6.4a, the total duodenal AA flow per kg DMI relative to silage DM concentration shows that all underlying random regression lines support the overall regression line resulting in a P-value of 0.079. This emphasises, that the measured responses to changes in silage DM concentration can be considered as linear in the entire range evaluated. Therefore, the effects shown in Paper III and IV can be obtained by increasing silage DM concentration independent of point of origin. Apparent rumen NDF digestibility was not affected linearly by silage DM concentration as illustrated in Figure 6.4b (P = 0.598), but potential quadratic effects were not possible to test, as only two levels of DM concentration were available within cut.

## 6.6 Increasing DM concentration – profit and challenges

Experiment 2 showed that the supply of MP to lactating dairy cows increased with increasing silage DM concentration (Paper III), and this increase can be considered as linear within the evaluated range as discussed above. The absolute amount digested of all individual AA increased, but the increased DM concentration negatively affected the AA profile of digested AA in the small intestine, as proportions of lysine and histidine decreased (Paper IV). As discussed in Paper IV, the histidine proportion normally is low in grass-based diets and often is the first limiting AA in these diets (Vanhatalo *et al.*, 1999; Huhtanen *et al.*, 2002), thus a lower production response than expected based merely on total MP supply will probably appear when increasing silage DM concentration. However, this statement could not be tested in the current experiment, as no effect on milk production was observed, probably because the cows used in the experiment were in late lactation and milk yield probably was not restricted by the availability of AA. Even though the AA profile is negatively affected, increasing silage DM concentration will still be beneficially, as the amount of histidine digested in the small intestine per kg DMI was increased with 15%, when silage DM concentration was 700 g/kg compared to 350 g/kg. Thus, the cow is provided with a higher amount of AA, also the AA which might be first limiting. Therefore, based on an AA nutritional point of view, it is an advantage to increase forage DM concentration as much as possible before ensiling.

Increasing forage DM concentration will, however, give some management challenges. The first issue is the loss of crop material in the field during wilting. As described in section 2.3, leaf material dries faster than stem material, therefore leaf material is more prone to be lost, as the risk for loss increases with increased DM concentration. In forages with DM concentrations nearly 750 g/kg, about half of the leaf material can be lost during wilting, which reduces the CP concentration in the harvested material with 2-3%-units (Alli *et al.*, 1985). Tedding or swath inversion is usually performed during wilting to speed up the drying process and these operations can increase losses, especially of dry legume leaves as the attachment of leaves and stems are more fragile than in grasses (Rotz and Muck, 1994). Especially white clover leaves have a high risk of being lost, as they appear loosely in the forage mass with no attachment to a stem fraction. However, the loss by mechanical operations is highly affected by type of machinery used, and the adjustment of the machinery (Rotz and Muck,

1994). Additional losses occur during raking, and the loss increases with increasing DM concentration, but is also affected by swath thickness. A higher quantity of crop material in the swath reduces the percentage of DM lost by raking (Rotz and Muck, 1994). If there is a high DM loss in the field, and additional leaf material is lost by increasing forage DM concentration, a reduced amount of protein will be harvested from the field, which is not desirable. At farm level, it is difficult to determine the loss in the field, and the farmer has no tools to quantify the loss. If the farmers have to increase forage DM concentration before ensiling to increase MP concentration, it would be useful for them or their advisors to have a tool to quantify the loss, and thereby improve harvesting management. In Paper VI, the practicability of using changes in leaf:stem ratio as a tool to determine field losses was tested. This tool assumes, that only leaf material is lost in the field, which probably is not the case, thus the estimates obtained by the tool are the minimum losses, as the losses will be higher when stem material is lost as well. Another important prerequisite for reliable estimates is that all used samples are representative. The harvested fields in Paper VI consisted of single species only, whereby the fields were uniform in distribution. In grass-clover mixtures used in practical farming, the proportion of different species can vary within the field, making representative sampling more difficult. The varying distribution of species can be handled by taking larger samples, but larger sample sizes can be difficult to handle and increase the time needed for representative mass reduction. Another important issue, also discussed in Paper VI, is that the stubble height in the sample taken before mowing is equal to the stubble height set by the disc mower conditioner. However, if properly conducted, changes in leaf:stem ratio can be used to estimate field losses.

Another challenge regarding increased forage DM concentration is that high DM crops are more difficult to compress and the final silage is more prone to aerobic spoilage during the feed out period than forages with lower DM concentrations (Kung Jr., 2014). If the crop is not well compacted, it is difficult to establish an anaerobic environment, which is important for a successful ensiling process. Furthermore, air can penetrate more easily into the silage mass during the feed out period, when the sealing is removed, giving rise to growth of aerobic microorganisms. In well fermented silage, the low pH and the acetic acid inhibit the growth of many aerobe microorganisms, whereby only some yeasts that are able to grow at low pH will start to grow when the silage is exposed to air (Muck, 2010). The growth of other aerobe microorganisms will first begin, when pH increases. The pH rises as the yeasts consume the lactic acid. In silage with a high DM concentration, the microbial fermentation is restricted during the ensiling process (section 2.3.1), and the final silage will have a higher pH and a lower concentration of fermentation acids (Paper III, Table 1). Therefore, the growth of many aerobe microorganisms will not be restricted and they will start to growth soon after the silage is exposed to air. Furthermore, a higher concentration of sugar is left in high DM silage compared to low DM silage (Paper III, Table 1) giving more substrates for microbial growth. The aerobe microbial growth generates heat, and high DM silage will heat up more easily than low DM silage due to a lower heat capacity. Heat can catalyse Millard reactions, which are chemical reactions between carbohydrates and proteins. Silages that have undergone Millard reactions have a lower digestibility of both protein and energy (Weiss *et al.*, 2003). Therefore, silage with a high DM concentration is less aerobically stable

than silage with a lower DM concentration. To overcome the challenge with reduced aerobe stability, good management in the feed out period is needed, especially when ensiling in bunker silos. The challenge with reduced aerobic stability can also be handled by ensiling in bales instead of in bunker silos, as the baling machine compresses high DM forage better than a loader in a bunker silo and a bale can be used within a day or two after opening (Ohlsson, 1998). However, depending on crop yields, labour costs, machine capacity, herd size etc. the costs can be higher for baled silage than for ensiling in a bunker silo (Ohlsson, 1998), but if it is possible to economise on purchased protein it can possible still be an advantage. As the positive effect of silage DM concentration on MP supply was linear, it is not necessary to increase silage DM concentration to 700 g/kg to get an advantage. Increasing silage DM concentration from e.g. 300 to 450 g/kg will be advisable and this also reduces the challenges, compared to increasing silage DM concentration to 700 g/kg.

## 6.7 Feed evaluation methods

In experimental feed evaluation, it is important to have analytical methods that can be used to screen and determine the value of a large range of feeds, without being too expensive or demanding. Differences between feeds obtained using these analyses preferably should reflect the difference in animal responses observed when feeding the actual feedstuffs. In Paper V, values for rumen protein degradation obtained using the *in situ* technique were compared with the actual rumen protein degradation measured *in vivo*. Even though the absolute values were quite different between the two measurements, probably due to a large contribution of CP from endogenous sources in duodenal flow *in vivo*, as discussed in Paper V, the decrease in rumen protein degradation when increasing silage DM concentration was similar for the two methods. Therefore, the *in situ* method seems to be an adequate method to examine differences in rumen protein degradation between grass-clover silages differing in DM concentration. However, the *in situ* analysis is normally used in experimental contexts and is not a standard analysis used in practical feed evaluation. The analyses used in practical farming have to be cheap and fast, therefore, near-infrared spectroscopy (NIRS) is a widely used method today. Even though NIRS is widely used, it is dependent on reference methods for calibration, for which reason it is still necessary to have good reference methods that describe what is actually happening in the animal.

In feed evaluation systems e.g. the Nordic feed evaluation system NorFor (Volden, 2011b), many animal responses are estimated based on chemical analyses of feeds and animal characteristics. Therefore, it is important that these estimations reflect what happens *in vivo*, when they are used to optimise feed rations. Hauge *et al.* (2015) estimated rumen microbial protein synthesis in NorFor based on the chemical composition of the eight silages used in Experiment 2. These estimated values were compared with the microbial protein synthesis measured *in vivo,* and Hauge *et al.* (2015) concluded that changes in rumen microbial protein synthesis affected by silage DM concentration were well detected by the NorFor estimates. This shows that the analyses and feed evaluation system used today can al-

ready detect the differences in protein quality between grass-clover silages pre-wilted to different DM concentrations. Therefore, the applicability of increasing silage DM concentration is larger than if the changes could not be handled by the used feed evaluation system.

# 7 Conclusion

This thesis contributed with improved knowledge on feeding and protein value of green forages. Based on the meta-analysis and Experiment 1, it is concluded that feed intake and milk production are higher in dairy cows fed legume-based diets than in dairy cows fed grass-based diets when forage OM digestibility is similar. Differences in milk production within different grass or clover species could be explained by differences in silage OM digestibility. However, the results indicated that there is an optimum for silage OM digestibility regarding milk production in the range 80-82%. Experiment 1 showed, that dairy cows fed grass silage with an OM digestibility above this optimum did not produce the expected amount of milk based on the amount of OM actually digested in the gastrointestinal tract. Furthermore, the results indicated that feed intake was regulated physiologically instead of physically, when dairy cows were fed clover silage with an OM digestibility above the optimal range. Therefore, farmers must consider inclusion rate of clover and digestibility, besides crop yields and production costs, to optimise profitability, and hereon select the species most suitable for local conditions.

Based on Experiment 2, it is concluded that MP supply to lactating dairy cows can be increased by increasing DM concentration in grass-clover before ensiling, without affecting NDF digestibility. The observed increase in MP supply was caused by an increased rumen microbial synthesis, a reduced rumen degradation of feed protein, and an increased small intestinal digestibility of AA. However, increasing silage DM concentration negatively affected AA profile of MP, as proportions of lysine and histidine in digested AA were reduced. Presumably, a lower production response than expected based solely on total MP supply will consequently appear when increasing silage DM concentration, as either histidine or lysine often are the first limiting AA in grass-based diets. However, the supply of all individual AA including those which might be first limiting was increased with increased silage DM concentration. Therefore, based on an AA nutritional point of view, it is beneficial to increase forage DM concentration as much as possible before ensiling. Increasing forage DM concentration to high levels can give some management challenges such as increased field losses and reduced aerobe stability of silage. The current results indicated that the positive effect of silage DM concentration on MP supply was linear, and therefore higher MP supply can be achieved also when increasing silage DM concentration from e.g. 300 to 450 g/kg.

Finally, it is concluded that the *in situ* technique seems to be an adequate method to detect differences in rumen protein degradation between grass-clover silages with different DM concentrations, and that changes in leaf:stem ratio can be used to estimate field losses if the plant material, collected in different steps of the harvesting process, is representative.

# 8 Perspectives

For a combined optimisation of forage and milk production, it is important to have knowledge on both cultivation characteristics and feeding value of different forage species. However, it is also important to know the combined effect, when green forage species are cultivated together or fed in combination with other feedstuffs. The current thesis confirms that milk production is higher when cows are fed legume-based diets compared to grass-based diets. However, marginal responses in milk production for increased clover proportion and thereby the most optimal inclusion rate of legume in the diet is unknown. More knowledge on this topic is needed, thus farmers can manage their grass-clover fields more optimal in relation to yield and clover proportion. Nitrogen fertilisation negatively affects the clover proportion but can increase the yield, as the grass become more competitive. However, it is difficult to study the isolated effect of clover proportion on milk production, as forage digestibility, which also affect milk production, often change with changed clover proportion.

The average OM digestibility in conventional grass-clover silages harvested in Denmark in 2016 was 73.7-76.6% in five cuts, however, the variation in OM digestibility within cut was approximately 60-83% (Thøgersen and Kjeldsen, 2017). The results from the current thesis indicate, that a higher OM digestibility is advisable to optimise milk production. The average OM digestibility in grass-clover silages should be increased by improving the OM digestibility in these silages that otherwise will be below the current average, thus reducing the overall variation in silage OM digestibility. The farmers that already produce silages with OM digestibilities around 80% will probably not have advantages of increasing silage OM digestibility further, as the current results indicated that there is an optimum for silage OM digestibility regarding milk production in the range 80-82%.

The current thesis showed, that MP supply to lactating dairy cows increased with increasing silage DM concentration. This response was measured in low yielding cows in late lactation fed only grass-clover silage, and therefore, the effect on milk production was not studied. It is assumed, that increasing silage DM concentration will have a positive effect on milk production, as the amount of available AA is a major factor affecting milk production. However, this has to be studied in dairy cows expected to respond on MP supply e.g. high yielding cows in early lactation. Furthermore, possible interactions when concentrate, maize silage or both are included in diet have to be studied as well, as mixed diets are used in practical farming. Even though the effect on milk production is not studied, the results from the current thesis regarding MP supply are promising, and therefore it is recommended, that forage DM concentration is increased to 400-500 g/kg from the current recommendation of 300-350 g/kg in Denmark (Nielsen *et al.*, 2003). At this higher DM concentration, the silage should still without difficulty be ensiled in bunker silos, however, it is important to be aware of the challenges (field losses and aerobe stability) when increasing forage DM concentration.

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