Grass for dairy cows

Fresh grass or silage from physically processed grass as alternative to common grass silage

PhD thesis
Nikolaj Peder Hansen
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Department of Animal Science
Faculty of Technical Sciences
Aarhus University

Main supervisor:

Professor, Martin Riis Weisbjerg
Department of Animal Science, Faculty of Technical Sciences, Aarhus University, Denmark

Co-supervisor:

Professor, Søren Krogh Jensen
Department of Animal Science, Faculty of Technical Sciences, Aarhus University, Denmark

Senior scientist, Troels Kristensen
Department of Agroecology, Faculty of Technical Sciences, Aarhus University, Denmark

Assessment committee:

Senior scientist, Ole Højberg (Chairman)
Department of Animal Science, Faculty of Technical Sciences, Aarhus University, Denmark

Research Professor, Håvard Steinshamn
Division of Production and Society, Norwegian Institute of Bioeconomy Research (NIBIO), Norway.

Professor, Jon Moorby
Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Wales.

Preface

The present thesis entitled “Grass for dairy cows - Fresh grass or silage from physically processed grass as alternative to common grass silage” was submitted to the Graduate school of Technical Sciences (GSTS) to obtain the PhD degree. The research related to the reported results was conducted between October 2018 and January 2022 at the Department of Animal Science, Aarhus University.

The PhD project was comprised of three projects: “GRÆSMÆLK” (English: GRASSMILK), funded by the Danish Milk Levy Fund and the Fund for Organic Farming; “BEGROME – Bedre grovfoder med mekanisk behandling” (English: BEGROME – better forage with mechanical processing), funded by the Danish Ministry of Food, Agriculture, and Fisheries; and “Optimal udnyttelse af bioraffineret pulp fra grøn biomasse til kvægfoder” (English: Optimal utilisation of biorefined pulp of green biomass as cattle feed), funded by the INTERREG project BIOCAS100 and the Hofmansgave Foundation. Moreover, funding was received from GSTS and the Center for Circular Bioeconomy (Aarhus University).

The objectives of this PhD project were to investigate the effects of feeding grass harvested at different developmental stages as either fresh or as silage from physically processed grass on digestibility of fibre, feed intake, milk production, and methane emission. The project contributes with documentation on milk production and methane emission in different production systems, and provides new knowledge regarding the effects of physical processing of forages on nutrient digestion. The knowledge was obtained through a production trial, where 16 dairy cows were fed fresh grass in the barn, and through two intensive feeding studies, where four and six multi-fistulated dairy cows were fed silages processed physically using two different methods.

The current thesis presents background knowledge on grass, methods for physically processing forage, and grass utilisation in dairy cows (Chapter 2), states the aim and hypotheses of the PhD project (Chapter 3), discusses the methodological approach of the three experiments (Chapter 4), presents the results of the three studies via five papers (Chapter 5), discusses the results of the experiments in accordance to existing literature and applicability (Chapter 6), and states the conclusions (Chapter 7) and perspectives (Chapter 8) of the PhD project.

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Contents

Preface ... iii
Acknowledgement ... iv
Summary ... vi
Sammendrag ... vii
List of included papers ... viii
List of other scientific publications ... ix
Abbreviations ... x

1 Introduction ... 1

2 Background ... 3

2.1 Grass – structure and growth ... 3
2.2 Processing of forage ... 6
2.3 Ensiling ... 10
2.4 Digestion and intake in ruminants ... 12
2.5 Production of methane and milk ... 17

3 Aim and hypotheses ... 19

4 Methodology ... 20

4.1 Study design ... 20
4.2 Digestibility and rumen kinetics ... 21
4.3 Feeding management ... 22

5 Results ... 23

5.1 Paper I - Effect of regrowth period for perennial ryegrass on yield and nutritive value of grass ... 25
5.2 Paper II - Effects on feed intake, milk production and methane emission in dairy cows fed silage or fresh grass harvested at early or late development stage with and without concentrate ... 29
5.3 Paper III - Shredding of grass-clover before ensiling: Effects on feed intake, digestibility, and methane production in dairy cows ... 73
5.4 Paper IV - Effect of screw pressing and days of regrowth on grass silage characteristics and quality ... 89
5.5 Paper V - Wet fractionation by screw pressing of grass into pulp and juice can improve fiber digestibility and protein value of pulp for lactating dairy cows ... 93

6 General discussion ... 135

6.1 Changes in nutrient composition and digestibility of grass during its development ... 135
6.2 Effects of physical processing and interactions with developmental stage ... 138
6.3 Methane emission ... 143
6.4 Milk production in cows fed high proportions of grass ... 145

7 Conclusion ... 147

8 Perspectives ... 148

9 References ... 149

Summary

Cultivating grass or grass-clover instead of maize has beneficial effects on the environment. The thesis presents methods aiming at improving the utilisation of grass and grass-clover in dairy cows. Three feeding studies contributed with documentation on milk production and methane emission in different production systems and provided new knowledge regarding the effects of physical processing of forages on nutrient digestion.

In the Fresh-Study, 16 second lactation dairy cows in mid-lactation were used in a cross-over design with two periods. Four dietary treatments were fed in the barn and varied according to forage type (fresh grass vs. silage), concentrate supplementation (0 vs. 6 kg/day), and developmental stage of fresh grass at harvest (early vs. late). The main response variables were changes in the nutrient composition of fresh grass during development (reported in Paper I) and DMI, milk production, and methane emission (reported in Paper II). In the Shred-Study, four rumen, duodenal, and ileal cannulated first lactation cows in late-lactation were used in a 4×4 Latin square design with four periods and a 2×2 factorial arrangement. The factors were developmental stage of grass-clover at harvest (early vs. late) and physical processing (shredded vs. non-shredded) of grass-clover prior to ensiling. The main response variables were fibre digestibility, fibre kinetics in the rumen, and methane emission (reported in Paper III). In the Pulp-Study, six multiparous and rumen, duodenal, and ileal cannulated cows in mid to late-lactation were used in an incomplete 6×4 Latin square design with four periods and a 2×3 factorial arrangement. The factors were developmental stage of grass at harvest (early vs. late) and physical processing of the grass prior to ensiling (chopped grass, pulp from one fractionation of grass, or pulp from two fractionations of grass). The main response variables were ensiling characteristics (reported in Paper IV) and fibre digestibility, fibre kinetics in the rumen, protein value of the silages, and methane emission (reported in Paper V).

It was concluded that milk yield, unexpectedly, was higher when cows were fed silage compared to fresh grass harvested at late developmental stage, whereas dry matter intake (**DMI**) and energy-corrected milk yield did not differ despite large numeric differences were observed. The effects were probably caused by a lower apparent total tract digestibility (**ATTD**) of organic matter (**OM**) of fresh grass compared to silage. Shredding resulted in decreased ATTD of neutral detergent fibre (**NDF**), whereas, at late developmental stage, ruminal and ATTD of NDF were higher for pulp pressed twice compared to chopped grass. At early developmental stage, the protein value was higher for pulp pressed twice compared to chopped grass, but NDF digestibility was not different. Shredding had no effect on DMI, whereas cows fed pulp had lower DMI but higher NDF intake compared to cows fed chopped grass. Feeding grass or grass-clover harvested at early compared to late developmental stage only reduced methane yield (L/kg of DMI) in the Pulp-Study and had no effect in the two other studies. However, shredding or feeding grass-clover harvested at late developmental stage reduced methane emission expressed as L/kg of OM digested in the rumen in the Shred-Study. The methane yield was higher for cows fed pulp pressed twice compared to chopped grass when comparing treatments of grass harvested at early, but not late developmental stage.

The thesis indicates that milk yield might not differ between cows fed silage or fresh grass if digestibility of OM of forages are similar and that physical processing has the potential to improve utilisation of grass, but that the results vary significantly.

Sammendrag

Dyrkning af græs og kløvergræs har positive effekter på miljøet. Denne afhandling præsenterer metoder, hvorpå malkekøers udnyttelse af netop græs og kløvergræs kan forbedres. Tre fodringsforsøg bidrog med dokumentation for effekter på mælkeproduktion og metanemission med forskellige fodringssystemer, og gav ny viden om effekterne af fysisk behandling af grovfoder på fordøjelighed af næringsstoffer.

I forsøg 1 blev 16 anden-kalvs køer i midt-laktation anvendt i et overkrydsningsforsøg med to perioder á 21 dages varighed. Fire typer behandlinger blev udfodret på stald og varierede i grovfodertype (friskt græs kontra ensilage), kraftfodertildeling (0 kontra 6 kg/dag) og udviklingstrinnet af det friske græs ved høst (tidligt kontra sent). De primære responsparametre omfattede udviklingsmæssige ændringer i næringsstofsammensætningen af frisk græs (rapporteret i Paper I) samt optag af tørstof (**TS**), mælkeproduktion, og metanemission (rapporteret i Paper III). I forsøg 2 blev fire multi-fistulerede første-kalvs køer brugt i et 4 × 4 romerkvadratforsøg med 4 perioder á 21 dages varighed og med et 2 × 2 faktorielt design. Faktorerne var udviklingstrin af kløvergræsset ved høst (tidligt kontra sent) og fysisk processering (shredded kontra ikke shredded) af kløvergræs inden ensilering. De primære responsparametre omfattede fiberfordøjelighed, fiberkinetik i vommen og metanemission (rapporteret i Paper III). I forsøg 3 blev seks multi-fistulerede anden-kalvs eller ældre køer i midt- til sen-laktationen brugt i et ufuldstændigt 6 × 4 romerkvadratforsøg med fire perioder á 21 dages varighed og med et 2 × 3 faktorielt design. Faktorerne var udviklingstrin af græs ved høst (tidligt kontra sent) og fysisk processering (snittet græs, pulp af græs efter én separering eller pulp af græs efter to separeringer) inden ensilering. De primære responsparametre omfattede ensileringskarakteristika (rapporteret i Paper IV) samt fiberfordøjelighed, fiberkinetik i vommen, ensilagens proteinværdi og metanemission (rapporteret i Paper V).

Mod forventning var mælkeydelsen højere for køer fodret med ensilage sammenlignet med friskt græs høstet på et sent udviklingstrin. TS-optaget og den energikorrigerede mælkeydelse var ikke forskellig mellem de to behandlinger til trods for væsentlige numeriske forskelle. Effekten skyldtes formentlig lavere tilsyneladende total fordøjelighed af organisk stof (**OS**) af friskt græs sammenlignet med ensilage. Shredning sænkede total fordøjelighed af fibre (**NDF**), hvorimod at der for det sene udviklingstrin var højere vom- og totalfordøjelighed af NDF for pulp presset to gange sammenlignet med snittet græs. Ved fodring med græs høstet på et tidligt udviklingstrin havde pulp presset to gange højere proteinværdi sammenlignet med snittet græs, hvorimod fordøjeligheden af NDF var upåvirket. Shredning havde ingen effekt på TS-optaget, hvorimod fodring med pulp presset to gange reducerede TS-optaget og øgede NDF-optaget sammenlignet med snittet græs. Fodring af græs eller kløvergræs høstet på tidligt sammenlignet med sent udviklingstrin reducerede metanproduktionen (L/kg TS-optag) i det tredje forsøg, og havde ingen effekt i de øvrige forsøg. Shredning eller fodring med kløvergræs høstet på sent udviklingstrin reducerede metanemissionen (udtrykt som L/kg OS fordøjet i vommen) i forsøg 2. Ved fodring med græs høstet på et tidligt udviklingstrin var metanproduktionen højere for køer fodret med pulp presset to gange sammenlignet med snittet græs.

Afhandlingen indikerer, at mælkeydelsen tilsyneladende ikke er forskellig mellem køer fodret med friskt græs og ensilage, når OS-fordøjeligheden er ens, samt at fysisk processering har potentialet for at øge udnyttelsen af græs, trods det at effekterne varierer meget.

List of included papers

I. **Hansen, N. P.**, T. Kristensen, M. Johansen, and M. R. Weisbjerg.
Effect of regrowth period for perennial ryegrass on yield and nutritive value of grass.
The Proceedings of the 28th General Meeting of the European Grassland Federation, 2020, Page 267-269. Helsinki, Finland.

II. **Hansen, N. P.**, T. Kristensen, M. Johansen, L. Wiking, N. A. Poulsen, A. L. F. Hellwing, L. Foldager, S. K. Jensen, L. B. Larsen, and M. R. Weisbjerg.
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III. **Hansen, N. P.**, T. Kristensen, M. Johansen, A. L. F. Hellwing, P. Waldemar, and M. R. Weisbjerg.
Shredding of grass-clover before ensiling: Effects on feed intake, digestibility, and methane production in dairy cows.
Animal Feed Science and Technology. 2021. 282:115124.

IV. **Hansen, N. P.**, J. Bitsch, S. K. Jensen, M. R. Weisbjerg, M. Ambye-Jensen, and M. Johansen.
Effect of screw pressing and days of regrowth on grass silage characteristics and quality.
The Proceedings of the 28th General Meeting of the European Grassland Federation, 2020, Page 620-622. Helsinki, Finland.

V. **Hansen, N. P.**, S. K. Jensen, M. Johansen, A. L. F. Hellwing, M. Ambye-Jensen, M. Larsen, and M. R. Weisbjerg.
Fiber digestibility and protein value of pulp silage for lactating dairy cows – effects of wet fractionation by screw pressing of perennial ryegrass.
Manuscript submitted (21-01-2022)

List of other scientific publications

Hansen, N. P., M. R. Weisbjerg, M. Johansen, and S. K. Jensen. 2021. Digestibility of grass fibre increased by screw pressing in a bio-refinery. Page 336 in the Book of abstracts of the 72nd annual meeting of the European federation of animal science. Davos, Switzerland.

Hansen, N. P., M. Johansen, L. Wiking, M. Larsen, P. Lund, T. Larsen, and M. R. Weisbjerg. 2021. Fava beans can substitute soybean meal and rapeseed meal as protein source in diets for lactating dairy cows. Journal of Dairy Science. 104:5508-5521.

Larsen, M., **N. P. Hansen**, M. R. Weisbjerg, and P. Lund. 2020. Technical note: Evaluation of the ororuminal FLORA device for rumen fluid sampling in intact cattle. Journal of Dairy Science. 103:447-450.

Hansen, N. P., P. Lund, M. R. Weisbjerg, and M. Larsen. 2019. Using the oro-ruminal FLORA device, volatile fatty acid proportions but not concentrations and pH were valid in rumen fluid samples. Page 639 in The Proceedings of the XIIIth International Symposium on Ruminant Physiology 2019. Leipzig, Germany.

Hansen, N. P., T. Kristensen, P. Waldemar, and M. R. Weisbjerg. 2019. Effect of harvest time and shredding of grass-clover on feed intake and chewing time in dairy cows. Page 554 in The Proceedings of the XIIIth International Symposium on Ruminant Physiology 2019. Leipzig, Germany.

Hansen, N. P., M. Johansen, M. R. Weisbjerg. 2018. Faba beans can replace soybean meal and rapeseed meal in diets for dairy cows. Page 580 in the Proceedings of the Xth International Symposium on the Nutrition of Herbivores 2018. Clermont-Ferrand, France.

Abbreviations

ADF	Acid detergent fibre
ADL	Acid detergent lignin
AA	Amino acid
AAT	Amino acids absorbed in the small intestine
ATTD	Apparent total tract digestibility
CH_4	Methane
CO_2	Carbon dioxide
CP	Crude protein
Cr_2O_3	Chromium oxide
DNDF	Digestible neutral detergent fibre
DM	Dry matter
DMI	Dry matter intake
EPD	Effective protein degradability in the rumen
ECM	Energy-corrected milk
k_d	Fractional rate of digestion
k_p	Fractional rate of passage
FSG	Functional specific gravity
iNDF	Indigestible neutral detergent fibre
LAB	Lactic acid bacteria
MP	Metabolisable protein
NDF	Neutral detergent fibre
NPN	Non-protein N
OM	Organic matter
OMD	Organic matter digestibility
PBV	Protein balance in the rumen
RUP	Rumen-undegraded protein
TiO_2	Titanium dioxide
TMR	Total mixed ration
VFA	Volatile fatty acids
WSC	Water-soluble carbohydrates

1 Introduction

Grass, grass-clover, and maize constitute the major part of forages cultivated for cattle feed in Denmark, and in 2021, approximately 278,000 ha of arable land was cultivated with grass-clover in Denmark (Statistics Denmark, 2022). Compared to most annual grain crops, the inclusion of perennial forages (such as grass, clover, and lucerne) in the crop rotation results in lower N leaching (Manevski et al., 2018; Børgesen et al., 2020), marginally higher C sequestration (Taghizadeh-Toosi and Olesen, 2016), and less use of pesticides (Jørgensen et al., 2021). Moreover, from an energy efficiency point of view, perennial forages utilise solar energy for a longer period of the year compared to annual grain crops (Manevski et al., 2017), and high CP yields are achieved in forages (Wilkins and Jones, 2000). The improved environmental effects of cultivating perennial forages are currently driving the political interest in increasing the proportion of forages, such as grass, in the crop rotation. Therefore, improving the utilisation of grass in dairy cattle nutrition is warranted.

Ruminants have a unique ability to utilise forage, which is difficult to digest for monogastric animal species. The symbiotic effects between the cow and the microbial community occupying the rumen of the cow enable the cow to produce milk from low-quality feedstuffs (McDonald et al., 2011). However, the constantly improving genetic potential for milk yield requires that the feed quality, even for ruminants, is optimised to reach the capacity for milk production. The milk production is affected by dry matter (**DM**) intake (**DMI**), organic matter digestibility (**OMD**), and thereby the energy intake (Allen, 1996b; Huhtanen and Nousiainen, 2012), and especially the concentration and digestibility of fibre (often termed neutral detergent fibre; **NDF**) affects the OMD, since NDF is digested slowly in the rumen. Diets high in NDF require longer retention time in rumen to enable the rumen microorganisms to adhere and colonize the feed particles and degrade the NDF, whereas concentrates, which typically have a low concentration of NDF, are more easily digested and have a high energy density (Krämer et al., 2013). Improving the digestibility and thereby the quality of grass will ensure that dairy farmers will be less dependent on securing sufficient supply of concentrates at prices that fluctuate due to the competitive use of concentrates in other agricultural sectors such as the pig or poultry industry. Moreover, the abovementioned beneficial effects from higher inclusion of perennial forages in the crop rotation are attained if the intake of forage can be increased without impeding the milk production.

Farmers comply with the organic principles (IFOAM, 2020) in various production systems and they are motivated for producing organic milk for different reasons. Due to increasing consumer demand and due to a push by public organic actions plans (Jespersen et al., 2017), the proportion of organic milk produced in Denmark has increased from 1% in 1993 to nearly 13% in 2020 (Statistics Denmark, 2022). For the past few years, the concept of “Grassmilk” has gained much interest and is marketed by the organic dairy Thise (https://thise.dk/en/). The principles of the concept is to produce milk from cows fed a 100% grass and legume-based diet in order not to compete for feedstuffs, which can be used for human consumption or in diets for monogastrics. Moreover, direct cut harvest machines with high harvesting capacities have received renewed interest in Denmark. The machines can ensure a high supply of fresh grass-clover for cows kept in barns, a concept also termed zero-grazing (Holohan et al., 2021). However, and as argued by Kristensen et al. (2005), the concepts and systems still lack

knowledge and documentation on effects on milk yield and milk composition, feed efficiency, and methane emission under Danish conditions.

The ruminal digestibility of NDF from grass depends on the processes of digestion and passage of NDF out of the rumen. At high fractional passage rates (**k_p**), the particles are retained for a shorter time in the rumen, resulting in less digestion but a potentially higher DMI (Mertens, 1993, 1994). Increasing the fractional rate of digestion (**k_d**) of digestible NDF (**DNDF**) without affecting k_p will improve the digestibility of NDF (Allen and Mertens, 1988) and possibly the milk yield through improved OMD (Jensen et al., 2015). Such effects have been investigated in ruminants by applying different types of physical processing to forage during harvest (Savoie, 2001). Shredding is a type of physical processing that is more intense than conditioning and seeks to severely damage the particle structure and particle surface of forage (Broderick et al., 1999; Lehmann et al., 2017). The intention is to increase the surface area and the number of suitable entries into the forage particles for microbes (Hong et al., 1988). If the k_d of DNDF is increased, utilisation of grass for milk production might be improved, and shredding could therefore be a potential driver for increasing the proportion of forages in diets of dairy cows and in the crop rotations.

Biorefining of green biomasses has shown to be an alternative pathway towards increasing the amount of locally produced protein, which can substitute parts of the imported soybean meal from e.g. South America and Asia, but also to provide the beneficial effects of including more perennial forages in the crop rotation (Jørgensen et al., 2021). Biorefining of biomasses, which in Danish settings mainly concerns grass and legumes, is a concept where the biomass is harvested green, processed in a biorefinery using e.g. a screw press, yielding a protein-rich liquid and a fibre-rich pulp fraction (Pirie, 1978; Damborg et al., 2020). A protein concentrate can be harnessed from the juice fraction, and the concentrate can substitute soybean meal in diets for monogastrics (Stødkilde et al., 2019). However, to achieve the most sustainable production, the side-streams of other products from the biorefinery must be utilised efficiently. Approximately 65% of the DM from the biomass is recovered in the pulp (Damborg et al., 2020), and the pulp has shown potential as a feedstuff for ruminants (Damborg et al., 2019; Savonen et al., 2019). However, more recent work has also shown negative effects on milk production of feeding pulp compared to the whole plant (Sousa et al., 2022). Any changes in the feeding value of pulp might be attributed to changes in the chemical composition, but also the intensive disintegration of fibre particles, mediating rapid colonisation and degradation of feed particles (Hong et al., 1988; Chiquette et al., 1994). Improving the processing settings during screw pressing might increase crude protein (**CP**) yield in the juice and increase the disintegration of fibres and thereby NDF digestibility. Knowledge on the variation in feeding value of pulp is highly needed by farmers for optimisation of feed rations in practice, and to gain more insight into the effect of physical processing on fibre kinetics in the rumen.

Despite OMD of forages and the concentration of CP decrease when the grass develops (Wilson, 1994), the herbage yield will increase if the length of the regrowth period is extended. As indicated, various approaches can be made to potentially improve utilisation of grass by ruminants. Combining these approaches with harvest of forage at a later developmental stage might ensure high herbage yields and good quality forage with respect to digestibility. The main objectives of this PhD project were therefore to investigate the digestibility, feed intake, milk production, and methane emission for cows fed fresh grass and physically processed grass harvested at different developmental stages.

2 Background

This chapter describes the characteristics of grass growth, the methods of post-harvest physical processing of grass, and the post-harvest chemical changes of grass during ensiling. Moreover, ruminant digestion of carbohydrates and protein is outlined, and its overall effect on production of milk and methane is described. The main emphasis is put on grass, but knowledge on legumes such as clover is also included to obtain a broader understanding of the role of perennial forages and occasionally due to limited literature on grass.

2.1 Grass – structure and growth

Along with legumes such as clover and lucerne, grass comprises the green forage used extensively in ruminant nutrition. Grass belongs to the botanical family *Poaceae* and is a monocotyledonous plant (Nelson et al., 2020). Perennial ryegrass (*Lolium perenne* L.) is a cool-season grass with a C_3 photosynthesis system, whereas warm-season grasses have a C_4 photosynthesis system (Volenec and Nelson, 2020). The cool-season grasses have a lower optimum temperature for growth compared to warm-season grasses (20-25 vs. 35-38 °C, respectively), and the yield potential for perennial ryegrass is high in temperate regions (Moser and Hoveland, 1996). Perennial ryegrass is characterised as being highly digestible compared to both warm-season grasses and other cold-season grasses. However, perennial ryegrass is also less tolerant to drought, heat, cold winters, and insufficient drainage (Casler et al., 2020). Perennial ryegrass is often grown in mixtures with other grass species to complement each of their strengths, and also in mixtures with legumes (botanical family *Fabaceae*) such as clover, which can provide protein and increase the protein concentration of the forage (Van Keuren and Hoveland, 1985). The following sections in this chapter describe the structure of ryegrass from cellular to organ level with a focus on fibre (neutral detergent fibre; **NDF**) and address the developmental changes in structure and nutrient composition.

The cell wall

The plant cell is distinguished from the animal cell by its cell wall, which provides structural strength to the cell and define its size and shape (McNeil et al., 1984). All plant cells have a primary cell wall deposited prior to cell division, after which the cell wall can develop in size and composition. The primary cell wall is situated extracellularly on the plasma membrane and contains structural polysaccharide complexes, structural protein complexes, and phenolic compounds (McNeil et al., 1984). The polysaccharides are grouped into cellulose, hemicellulose, and pectin. Cellulose is a β-(1,4)-linked D-glucan chain, and several cellulose molecules can bundle into cellulose microfibrils (Cosgrove, 2005). The cellulose microfibrils are synthesised by complexes in the plasma membrane and then embedded in the cell wall in the matrix polysaccharides, also known as hemicelluloses. Cellulose is characterised by being insoluble in water, but soluble in a strong acid (Giger-Reverdin, 1995). The hemicelluloses are synthesised intracellularly and, when released into the cell wall, they associate with the newly synthesised cellulose microfibrils (Scheller and Ulvskov, 2010). Hemicelluloses provide strength to the cell wall by interacting with the cellulose microfibrils and, in some cell wall types also lignin. In grass, the main group of hemicellulose situated in the primary cell wall is xylans, a group of polysaccharides having a backbone of β-(1,4)-linked D-xylose residues, to

which mainly arabinose residues are attached (Scheller and Ulvskov, 2010). Hemicelluloses are insoluble in water but soluble in acid, except for β-glucan, which is extracted without acid. Finally, pectin is a diverse group of polysaccharides containing large amounts of galacturonic acid residues, and the concentration is generally higher in dicots compared to monocots, and highest in the primary cell wall and middle lamella compared to the secondary cell wall (Caffall and Mohnen, 2009). Just as β-glucan, pectin is easily solubilised in hot water.

When the growth of the primary cell wall ceases, some cell types have a secondary cell wall deposited and undergo lignification (Wilson, 1993; Taiz and Zeiger, 2003). The secondary cell wall develops from the inside of the primary cell wall and grows towards the middle of the cell and provides structural strength through resistance to tension and compressive force (Wilson, 1993). The secondary cell wall is composed of especially cellulose microfibrils, which are most often deposited in three different directions dividing the secondary cell wall into three layers (Wilson, 1993). Besides the presence of cellulose and hemicellulose, lignification occurs in some cells, but is highly dependent on forage type, tissue type, and environmental factors (Buxton and Russell, 1988; Wilson and Mertens, 1995). Lignin is an indigestible compound, comprising a large group of aromatic polymers composed of hydroxycinnamyl alcohols, and lignin deposition starts in the middle lamella and proceeds through the primary cell wall into the secondary cell wall (Vanholme et al., 2010). The lignin concentration is, therefore, highest in the middle lamella and the primary cell wall, whereas lignin in the secondary cell wall accounts for a major part of lignin in the whole cell due to the high volume taken up by the secondary cell wall (Wilson, 1993).

In the Nordic feed evaluation system NorFor (Volden, 2011), cell wall constituents are quantified chemically as NDF by boiling samples in a neutral detergent as described by Mertens (2002), which is a method originally developed by Van Soest (1963). However, pectin and β-glucan are recovered in the neutral detergent solubles rather than the NDF, showing that not all cell wall constituents are determined by the NDF analysis. Compared to grass, legumes have a high concentration of pectic substances, and therefore using the NDF procedure will underestimate the concentration of cell walls in legumes. The NDF concentration describes the concentration of fibres that are not readily solubilised in the rumen, which is better suited for feed evaluation in ruminants. The NDF is commonly said to account for hemicellulose, cellulose, and lignin. Boiling NDF in an acid solution solubilises hemicellulose, and the remaining fraction, acid detergent fibre (**ADF**), comprises cellulose and lignin. Treating ADF in a sulphuric solution solubilises cellulose, and the residual fraction is termed acid detergent lignin (**ADL**).

Plant tissues and plant organs used for forage

Overall, three major tissue types are present in the plant; dermal, vascular, and ground tissue. The physiochemical properties of each type of tissue differ due to differences in the composition of saccharides in various tissues (Nelson and Moore, 2020). The epidermal tissue is the outermost layer of the plant, and the cells in the epidermis have a thick cell wall, are lignified, and their outer surface is often covered with cuticle and wax (Wilson, 1993; Nelson and Moore, 2020). The latter is more pronounced in stems than in leaves and necessitates that these cells are chewed before microbes can penetrate the cell walls (Wilson, 1993). The inter-cellular linkages between epidermal cells in legumes and grasses are weakly lobed and straight-

sided, respectively, and the cells seem to split mainly along the middle lamella (Wilson, 1993). Vascular tissue appears both in stem, sheath, petiole, and leaf, and is comprised of phloem (nutrient conducting tissue) and xylem (water conducting cells) tissue (Mitchell et al., 2020). Cells in the xylem tissue have a thick cell wall and can be heavily lignified, whereas cells in the phloem tissue are thin-walled and do not lignify. The vascular tissue is arranged in vascular bundles in stem and leaf (called veins in leaf), and in legume leaves, the venation is more scattered, whereas it is straight-lined in grasses (Wilson, 1993; Mitchell et al., 2020). The remaining part of the tissue in the plant organs used for forage is made up of ground tissue of which mesophyll, parenchyma, collenchyma, and sclerenchyma are important tissue types. Mesophyll cells are abundant in leaves compared to stems, and are easily digested in grasses and legumes as they have a thin cell wall and do not lignify (Mitchell et al., 2020). Parenchyma is present in high concentration in sheath, stem, and petiole, and the tissue is easily digested in clover, whereas parenchyma in grasses develops a thick secondary cell wall and lignify (Mitchell et al., 2020). Collenchyma has an enlarged primary wall but does not lignify and is therefore also easily digested, whereas sclerenchyma cells develop a thick secondary cell wall and become lignified (Wilson, 1993). In clover, sclerenchyma is only found in small patches around the main vein in leaves and has only a small effect on digestibility, whereas sclerenchyma appears in grasses above and below vascular bundles in leaf and sheath (Wilson, 1993). In grass stems, sclerenchyma is associated with the vascular bundles or appears as a ring between the vascular bundles and the epidermis, providing mechanical strength for the plant (Wilson, 1993). Due to the lignification of sclerenchyma cells, the middle lamella and the primary cell wall are almost indigestible, whereas the secondary cell wall can be digested to some extent and the concentration of sclerenchyma cells plays an important role in reducing feed intake (Wilson, 1994).

The digestibility of different plant organs is related to the tissue type present in each organ. Wilson (1994) stated that the digestibility of the cell wall content overall ranged from 30 to 60%, but from 0 to 100% for individual cell types. Generally, the sheath and petiole can be considered as an intermediate of the stem and leaf with regard to the concentration of different tissue types, although it resembles stem more than leaf (Wilson, 1993). The stem proportion is generally negatively correlated to digestibility since clover stems have a high concentration of vascular tissue and grass stems have a high concentration of vascular tissue, sclerenchyma, and parenchyma in stems (Akin, 1989). In addition, epidermis, sclerenchyma, and parenchyma cells are more lignified in stems than in leaves (Akin, 1989). Finally, the main difference between grass and clover with respect to NDF digestion is which types of tissues lignify. In clover, xylem in vascular tissue in the stem is the only tissue that lignifies (Wilson, 1993). Conversely, in grass, lignification occurs in xylem, sclerenchyma, and parenchyma tissues in the entire plant (Wilson, 1993). Consequently, clover has a lower concentration of digestible NDF (**DNDF**), but a higher fractional rate of digestion ($\mathbf{k_d}$) of DNDF compared to grass (Wilson and Kennedy, 1996).

The main factor affecting plant morphology and digestibility is the plant maturity or developmental stage at harvest (Nelson and Moser, 1994). As the plant develops, the chemical composition of the whole plant and within plant organs changes due to increased stem proportion, since stems generally contain more cell walls than leaves. The proportion of different tissues in leaf, sheath, and petiole do not change as the plant develops, but the digestibility is reduced due to maturation of the cells, since cell walls will therefore comprise

a larger proportion compared to the cell solubles (Wilson, 1994). However, later-formed compared to early-formed leaves seem to have a higher concentration of vascular tissue and sclerenchyma (Wilson, 1993). In the stem, the proportion of vascular tissue in clover as well as vascular tissue, sclerenchyma, and parenchyma in grass constitute an increasing proportion as the plant develops, and all tissue types become less and less digestible (Akin, 1989). In general, cellulose and lignin will constitute a larger proportion as the plant develops. In the stem, parenchyma will develop a heavily lignified ring that surrounds the vascular bundles, and the concentration of lignin will be highest in the bottom of the stem for both grasses (Chen et al., 2002) and clovers (Wilson, 1993).

2.2 Processing of forage

Harvest of grass for ruminant feeding includes several mechanical actions in the field. The series of mechanical actions during or post-harvest are performed differently around the world, and both the method of each mechanical action and the objective of the mechanical action might differ. This section covers aspects of mechanical actions related to physical processing via precision chopping, shredding, and fractionation in a screw-press.

Precision chopping

Most commonly, green forages are mowed and left for wilting to increase the DM concentration prior to ensiling. The rate of wilting can be increased by tedding, and prior to harvest, the forage is raked into a narrow string, which can be picked up by one of several different types of harvesters. The principles of many of the harvesters are extensively described by Shinners (2003). In Denmark, most forages are harvested using a self-propelled harvester equipped with a cylindrical horizontally mounted cutterhead (Figure 2.1). The forage is lifted from the ground by a pick-up and channelled to the cutterhead via at least two feedrolls, where the forage is cut between the knives mounted on the cutterhead and the stationary shearbar. Several adjustments to the equipment can be made to improve the physical properties of the final forage product and to reduce energy costs (Shinners, 2003). The physical properties of the forage are widely described mainly using the expression theoretical length of cutting. The theoretical length of cutting is an expression derived mathematically using information about the diameter and rotational speed of the feedrolls, the diatmeter and rotational speed of the cutterhead, and the number of knives mounted on the cutterhead (Shinners, 2003). Despite a small particle size of the forage is mostly preferred, the fuel consumption of the self-propelled harvester increases when the theoretical length of cutting is reduced (Lyngvig, 2015).

Setting the equipment for a certain theoretical length of cutting results in forage having a distribution of particle lengths. The width of the distribution depends on the forage type (Shinners, 2003). The distribution for alfalfa and grasses is normally wider than that of maize, since upright cropped forages like maize are harvested such that the stem is channelled perpendicular towards the cutterhead. This produces a more uniform orientation of plant stem parts. In contrast, the stems and leaves of grass and alfalfa will have different orientations when the forage is channelled to the cutterhead; thus, some plant particles will face the cutterhead parallel rather than perpendicular producing forage cut with very long particle lengths (Shinners, 2003).

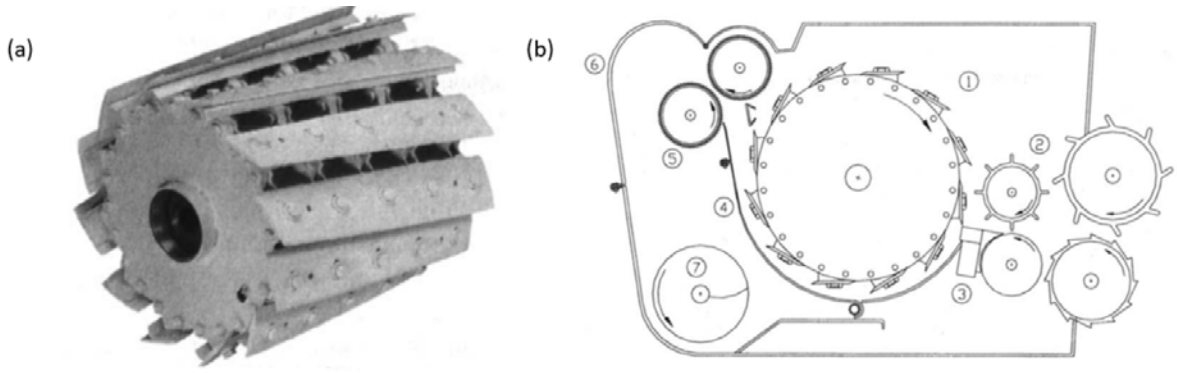

Figure 2.1: A) Cutterhead and B) schematic of a forage harvester with cutterhead (1), feedrolls (2), shearbar (3), removeable crop detector (4), and crop processing mill (5) (Shinners, 2003).

Shredding

The disc-mowers used for mowing grass are sometimes equipped with either a tine conditioner or a roller conditioner, which aim to crimp the harvested forage by breaking feed particles apart (Lehmann et al., 2017). To do so, the tine conditioner is a horizontally oriented cylinder, rotating at high speed and equipped with small v-shaped fingers capable of channelling the forage across a conditioning plate. The roller conditioner is comprised of two horizontally oriented rollers, between which the feed is channelled and processed due to the different surface patterns of the rollers. Shredding, an expression used intertwiningly with the term maceration, is a mechanical or physical process that seeks to crush and mash the forage particles to a much greater extent than conditioning. Further, shredding tears feed particles apart, whereas chopping cuts the feed particles (Savoie, 2001). Shredding was extensively studied in the late 1980's and throughout the 1990's by researchers in especially Canada and USA as reviewed by Savoie (2001). Most studies were performed on alfalfa and were intended to increase drying rate of the forages in the field, but have also focused on the effects on digestibility of e.g. ryegrass (Broderick et al., 2002).

The machine used for shredding, the shredder, can be used at several time points during harvest of the grass. The design of the shredder has changed throughout the years due to improvements in fuel economy and forage quality characteristics (Savoie, 2001; Descoteaux and Savoie, 2002; Samarasinghe et al., 2019). The principles of the early design of shredders are shown in Figure 2.2. Common for the three principles is that within each design, forage was channelled in between steel rollers with corrugated surfaces rotating at different speeds. The difference in speed between the rollers resulted in a shearing effect, where plant cells were ruptured and plant tissues and organs were torn apart. The drying rate of forages was probably increased since shredded forage particles were fractured, thereby increasing the surface from which water could evaporate. To increase the drying rate, the optimal time for use of the shredder is after the forage is mowed, either using a separate machine or using the same machine (as shown in Figure 2.2).

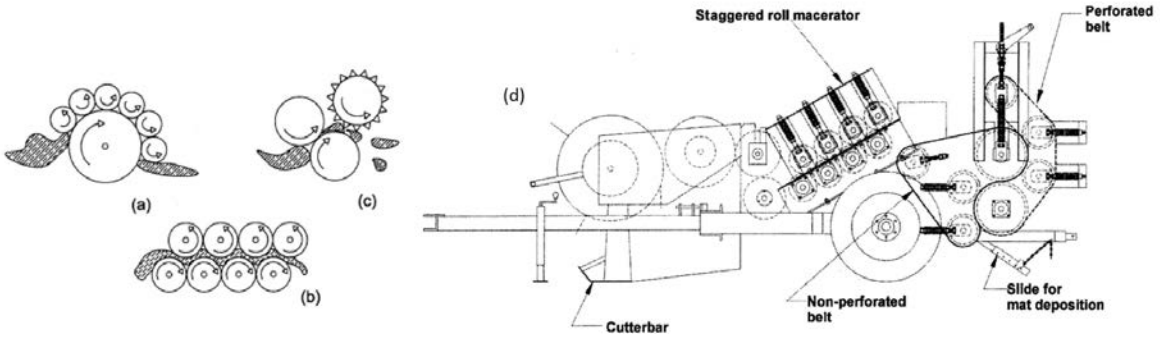

Figure 2.2: Principles of three early design shredders called A) peripheral roll macerator, B) staggered roll macerator, and C) crushing impact macerator, and D) a schematic of field prototype shredder with cutterbar mower (Savoie, 2001).

More recently, a laboratory scale machine with a new design for shredding was proposed by Samarasinghe et al. (2019) as shown in Figure 2.3. Rather than channelling forage in between two or more rollers, forage was channelled in between a roller and a curved shell, both with oppositly oriented steel ridges, and a larger processing area than the earlier designs.

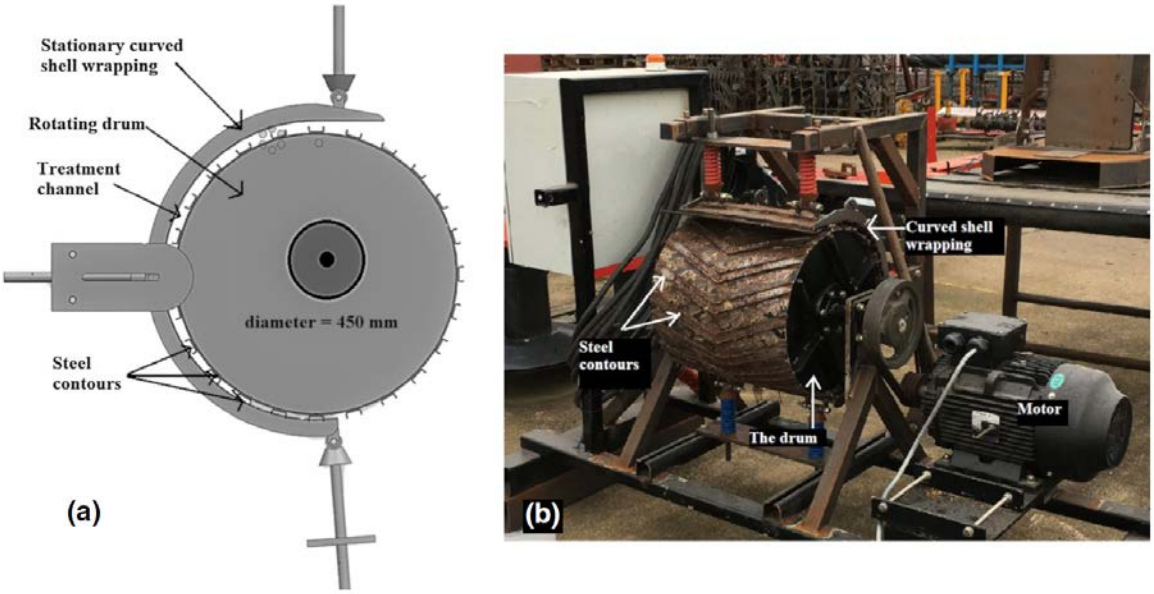

Figure 2.3: A) Schematic and B) photo of laboratory-scale shredder with rotating drum and stationary curved shell (Samarasinghe et al., 2019).

Fractionation using a screw press

Fractionation is a mechanical process that received much attention in the late 1970's (Pirie, 1978), and it is one of several processes performed in a biorefinery. Biorefining is a concept where raw materials, such as green forages, can be processed using different technologies to harness high-value products, while still utilising remaining side-streams of products (concept illustrated in Figure 2.4). In short, green forages, such as grass and legumes, are harvested from the field and transported to the biorefinery, where it is fractionated using e.g. a screw press. The fractionation results in a fibre-rich solid fraction, which can be utilised by ruminants, and

a liquid fraction rich in protein and other soluble compounds. The protein in the liquid fraction is precipitated by the application of heat or addition of acid. The protein is then separated and used as a protein concentrate for monogastric animals or intended for human consumption, while the residual liquid, the brown juice that is rich in minerals, can be utilised alongside manure from livestock for biogas production, and eventually be applied back on the fields (Pirie, 1978; McEniy and O' Kiely, 2014). In the rest of the thesis, emphasis is put on the effect of fractionation on the characteristics of the pulp.

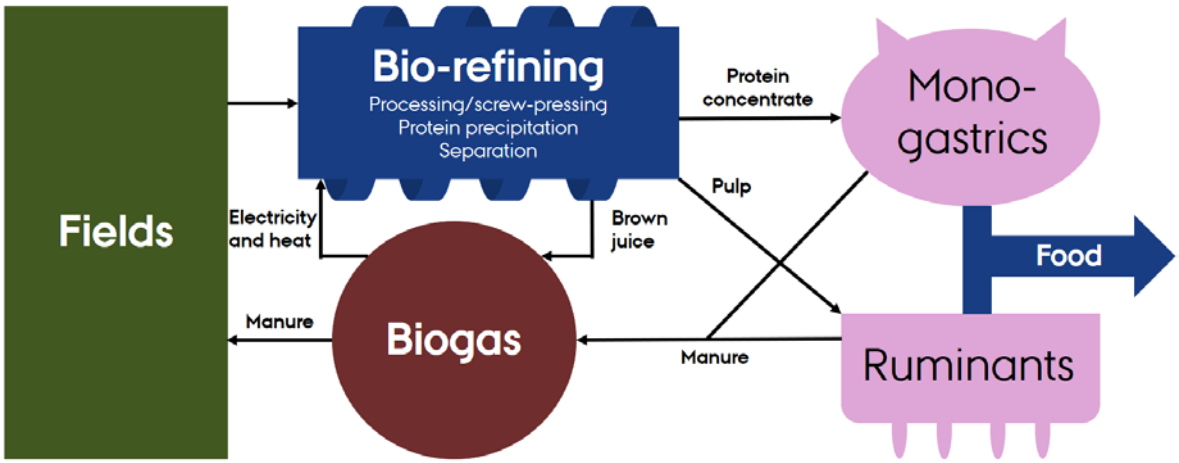

Figure 2.4: The concept of biorefining, where all side-streams are utilised.

The method of fractionation has changed through the years, but using a twin-screw press has shown great potential for protein extraction both in the laboratory (Colas et al., 2013) and in actual production (Santamaria-Fernandez et al., 2019). Regardless of size, the design of the twin-screw press is essentially the same. The device is composed of a barrel in which two parallel shafts are placed horizontally. The forage introduced into the barrel is drawn from one end of the shafts to the other when the shafts rotate since the surface of the shafts is comparable to an auger (Santamaria-Fernandez et al., 2019). The surface of the shafts changes appearance at the end to decrease the passage rate of forage thereby increasing the pressure. The high pressure disrupts plant organs and tissues and disintegrates the NDF structure of the cell walls, thereby releasing soluble nutrients from the plant cell into the free liquid (McEniy and O' Kiely, 2014). The liquid fraction is separated via sieve pores located in the barrel towards the end of the shafts. The solid fraction is termed pulp, and is used interchangeably with the term "press-cake".

There are several advantages of fractionating green forages and using the pulp as a feedstuff for ruminants. The forage does not need to be wilted prior to fractionation, meaning that the forage can be harvested in a direct cut with low DM concentrations, thereby reducing the risks of soil contamination and elevated ash concentrations due to raking and using a pick-up on the harvester. Furthermore, the intensive physical treatment during fractionation completely changes the physical appearance of the pulp compared to traditionally precision-chopped forage. To extract cellular solubles, the particles of pulp, down to cellular level, must have been damaged to some extent (Pirie, 1978, McEniy and O' Kiely, 2014). However, visual confirmation has not demonstrated this yet.

2.3 Ensiling

Forage for cattle is provided as either fresh herbage or preserved forages (e.g. as silage or hay). Forages are preserved in cattle nutrition to supply feed for the animals during times where herbage growth in the field is limited or non-existent. Prior to ensiling, factors such as forage type, harvesting technique, and season play a crucial role in potential DM losses and the preservation of energy and protein. Some of those factors also affect the fermentation pattern during ensiling.

Losses prior to ensiling

Generally, there are three causes of DM loss in the field; respiration, leaching during rain, and during mechanical handling (McGechan, 1989). The respiration loss might initially increase when forage is conditioned due to higher enzymatic activity, but the increased drying rate with conditioning counteracts this loss, as enzymatic activity is reduced with increasing DM concentration of the forage (Rotz and Muck, 1994). The DM loss in grass due to respiration often ranges between 2-5%, whereas the DM loss due to leaching during rain ranges between 1-27%, depending on the amount of rainfall (Rotz and Muck, 1994). The mechanical loss is associated with different mechanical actions in the field such as cutting, conditioning, shredding, tedding, raking, chopping, or baling, and raking of especially very dry forages might cause up to 20% loss of DM (Rotz and Muck, 1994). Leaves dry relatively fast compared to stems, and legume leaves detach easily from the stems, meaning that these are easily lost during raking (McGechan, 1989).

Ensiling

The nutritional changes occurring during the ensiling process concerns the formation of populations of microorganisms that ferment substrates into mainly preservative acids, and the changes are strongly related to the forage DM concentration, water-soluble carbohydrate (**WSC**) concentration, and buffer capacity (Wilkinson et al., 2003). After sealing the silo with compacted forage, the residual oxygen entrapped in the forage particles is used for respiration, resulting in an anaerobic environment (McDonald et al., 1991; Rooke and Hatfield, 2003). A desirable ensiling process is obtained with rapid production of a strong acid, lactic acid, and a concomitant drop in pH (McDonald et al., 1991). Homo- and heterofermentative lactic acid bacteria (**LAB**) produce lactic acid from hexoses of many types of WSC using several pathways (Pahlow et al., 2003). The homofermentative LAB ferment one molecule of hexose into two molecules of lactic acid only, which preserves all DM but reduces the energy concentration. However, the heterofermentative LAB ferment one molecule of hexose into one molecule of lactic acid and other molecules such as ethanol or acetic acid and mannitol. In the case of ethanol formation, substantial DM loss also occurs (Pahlow et al., 2003). In the early phase of ensiling, LAB competes with other microorganisms for substrates, and if a sufficiently low pH has not been reached early, populations of other organisms such as enterobacteria and clostridia will start taking over (Pahlow et al., 2003). The growth of enterobacteria results in great losses, whereas especially DM, but also energy, is lost from pathways of the clostridia (McDonald et al., 1991).

It is essential to reduce the DM concentration of the forage prior to ensiling to reduce the water activity since water activity is correlated to the level of pH at which the fermentation process stabilises (McDonald et al., 1991; Wilkinson and Davies, 2013). By increasing the DM concentration of the forage, less production of acid is required to reach the optimal pH for inhibiting the growth of undesired microorganisms. However, a DM concentration that is too high can result in greater DM losses during harvest. Therefore, achieving a DM concentration around 30-35% prior to ensiling is optimal for limiting the total loss (Muck et al., 2003). In forages with low concentrations of WSC such as legumes, less substrate is available for the initial ensiling phase, where lactic acid is produced and low pH is achieved (Rooke and Hatfield, 2003). Therefore, increasing the DM concentration of legumes prior to ensiling is imperative to avoid a secondary fermentation, where e.g. produced lactic acid is used by undesirable microorganisms (Pahlow et al., 2003). Shredding could potentially improve the initial ensiling phase since the cell wall structure is intended to be ruptured, which releases soluble nutrients faster, as indicated by e.g. the high rate of fermentation weight losses of shredded compared to untreated forages reported by Samarasinghe et al. (2019). Furthermore, the buffer capacity is generally higher in legumes compared to grasses (McDonald and Henderson, 1962), meaning that relatively more acid is required for lowering the pH in silage of legumes compared to grass. The higher buffering capacity is caused a by higher concentration of minerals and soluble crude protein (**CP**) (Rooke and Hatfield, 2003).

In silage, the volume is made up of DM, water, and air located in so-called air voids in the particles, and the silage bulk density (kg silage/m^3) and silage DM density (kg silage DM/m^3) influence the fermentation pattern and the aerobic stability of the silage when the silo is opened for feeding out (Muck et al., 2003). Increasing the silage DM concentration will increase the number of air voids and thereby decrease the silage bulk density, whereas increased DM concentration will increase the silage DM density, mainly due to reduced stiffness of fibres (Muck et al., 2003). Reducing the forage particle size has generally increased the silage DM density, but if feed particles become too small, nutrients can be lost via effluents (Muck et al., 2003). Shredding has been shown to increase the silage DM density (Shinners et al., 1988; Savoie et al., 1996; Samarasinghe et al., 2019), probably caused by the disintegration of the fibre structure. Silage density should be increased to reduce the porosity of the silage, which is directly proportional to the movement of oxygen into the silage (Muck et al., 2003). If the porosity is too high, aerobic microorganism such as yeasts and moulds will proliferate in the near area of the open end of the silo during feed-out. As shown in Figure 2.5, at a given DM concentration, porosity decreases with increasing silage density, which can be obtained by applying sufficient pressure from machinery or shredding the forage prior to ensiling. Moreover, reaching sufficiently high silage densities will reduce the amount of oxygen present in the forage at the onset of ensiling, and therefore, the formation of a desirable microbial community (mainly LAB) can start shortly after ensiling (Muck et al., 2003). Achieving a desirable fermentation pattern of the forage is achieved by a rapid increase in the lactic acid concentration and pH must be decreased quickly to a sufficiently low level. This depends on the silage DM and WSC concentrations, the buffer capacity, and the silage density (McDonald et al., 1991).

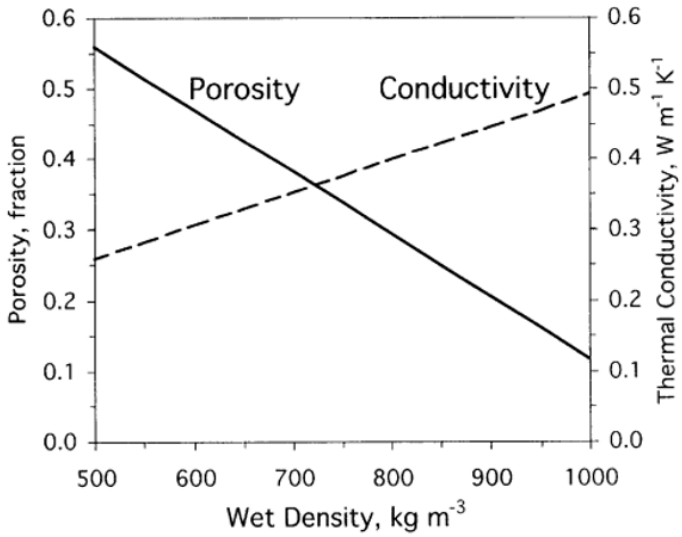

Figure 2.5: The effect of density on porosity and thermal conductivity in a silage at a fixed DM concentration of 35% (Muck et al., 2003).

2.4 Digestion and intake in ruminants

The digestive tract of the cow is composed of the mouth, oesophagus, rumen, reticulum, omasum, abomasum, small intestine, and large intestine (McDonald et al., 2011). In the mouth, feed particles are reduced in size during mastication while eating and ruminating, and the particles are mixed with saliva, containing buffers such as bicarbonate and phosphate (McDonald et al., 2011). Together, the rumen and reticulum comprise a large compartment, the reticulo-rumen, which acts as a large fermentation chamber. The reticulo-rumen is occupied by numerous different micro-organisms (bacteria, protozoa, archea, and fungi), which mediate the anaerobic degradation of nutrients, mainly carbohydrates and protein (Firkins, 2021). Extracellular enzymes excreted from microorganisms cleave cellulose and hemicelluloses into their monomers, which are metabolised intracellularly by the microorganisms, yielding mainly volatile fatty acids (**VFA**) and gases such as CO_2 and CH_4 (Janssen, 2010; Belanche et al., 2012). The microorganisms hydrolyse peptide bonds, deaminate AA, and utilize N from degraded feed N or recycled urea N from the saliva for the microbial protein synthesis (Clark et al., 1992; Firkins, 1996). Together with the majority of the produced VFA, excess N in the form of ammonia is absorbed through the rumen wall (France and Siddoons, 1993), and the extent of nutrient degradation in the rumen depends on both animal and feed characteristics (Okine et al., 1998). In the omasum, large amounts of water and some of the VFA are absorbed, and in the abomasum, HCl and pepsin are secreted resulting in acidification of the digesta. The small intestine is comprised of three sections, duodenum, jejunum, and ileum. When entering the small intestine, digesta is mixed with enzymes and bile, aiding to degrade nutrients into their monomers, enabling them to be absorbed in the small intestine (Lapierre et al., 2006). In the large intestine, comprised of the caecum and the colon, fermentation occurs as in the rumen, although produced VFA here constitute a minor proportion of total VFA absorption in the total tract, since most NDF is digested in the rumen (Huhtanen et al., 2006). Moreover, the protein from the microbial protein synthesis in the large intestine is wasted, as no absorption of AA occurs after the small intestine.

Fibre digestion and kinetics in the rumen

The unique ability of the cow to utilise NDF in feed for energy is caused by its evolutionary development of the rumen cavity, where microbial degradation and fermentation of NDF occur (McDonald et al., 2011). The forage particles with high concentrations of NDF require longer retention times in the rumen for microbes to colonise the particles and initiate the degradation. This is mediated by selective retention due to particle size and other factors as addressed in the following.

Rumen microbes adhere to plant particles and secrete cellulase extracellularly, which cleave cellulose into sugar monomers. The monomers are absorbed by the microorganisms and then fermented into VFA among other products. The type of VFA produced depends on the type of microorganism, and the VFA composition in the rumen liquid depends on the nutrient composition of the feed; thus, diets with high NDF concentrations generally yield a high proportion of acetate and high starch diets generally yield a high proportion of propionate (Janssen, 2010, Firkins, 2021).

The disappearance of NDF from the rumen occurs either by digestion or by passage out of the rumen and the rate of both actions affects digestibility and feed intake (Waldo, 1986). High rates of passage mediate higher feed intake but also reduces mean retention time in the rumen of DNDF, which decreases the digestibility of the feed (Allen and Mertens, 1988). Passage out of the rumen of particles is not random, and it has been suggested that reduction of particles to a critical particle size is required for enabling the particles to leave the rumen (Poppi et al., 1980). When reduced in size, particles have a probability of escaping through the reticulo-omasal orifice. The kinetics of digestion and passage can be modelled in various ways, and most typically, a two-pool model comprised of a non-escapable and an escapable pool is applied (Allen and Mertens, 1988). However, literature has stated several times that large particles have also escaped the rumen, and that properties such as particle shape or the liquid and gas entrapped in the cells affect the particle buoyancy (Shaver et al., 1988; Wilson and Kennedy, 1996). Physical parameters such as low functional specific gravity (**FSG**) seem to withhold particles in the non-escapable pool (Allen, 2000). The FSG is low in particles in the non-escapable pool since newly ingested particles have a high concentration of fermentable substrates compared to older particles. This is indicated by the higher NDF to indigestible NDF (**iNDF**) ratio of large compared to smaller particles in the rumen content (Allen, 2000). Gas is formed by the microbes, when the fermentable substrates are digested, and the gas bubbles adhering to the particles contribute to the particle's buoyancy (Wattiaux et al., 1992). When the proportion of digestible nutrients of the given particle has become low, less microbial activity and thereby gas production is associated with the particle, meaning that FSG increase and the particle sediments in the ventral part of the rumen. Here the probability of escape through the reticulo-omasal orifice is higher (Huhtanen et al., 2006).

Efficient microbial degradation of plant particles requires microbes to be in close proximity to the plant particles and that microbes have access to the interior tissue of the particles (Kennedy and Doyle, 1993). The cuticle layer on the outside of plant particles limits the adhesion of microbes to the intact plant tissue, and thereby also the digestion (Wilson and Mertens, 1995). Figure 2.6 shows the potential of microbial adhesion, as the surface of shredded lucerne stems was heavily colonised with microbes compared to the surface of the

same, but non-shredded plant. Hong et al. (1988) showed that colonisation occurred faster on surface areas of lucerne that had been cracked by shredding compared to intact plant surfaces. Despite the increase in colonisation of shredded forage, the reported effects of shredding on k_d of NDF and digestibility of NDF are inconsistent (Petit et al., 1994; Agbossamey et al., 2000; Broderick et al., 2002). The inconsistencies might be attributed to differences in forage type, type of shredder, etc. Microbial degradation has a limited direct effect on particle size reduction, which is mainly mediated by mastication during rumination, although microbial degradation indirectly affects particle size reduction by weakening the structural confirmation of the plant tissue through digestion (Kennedy and Doyle, 1993).

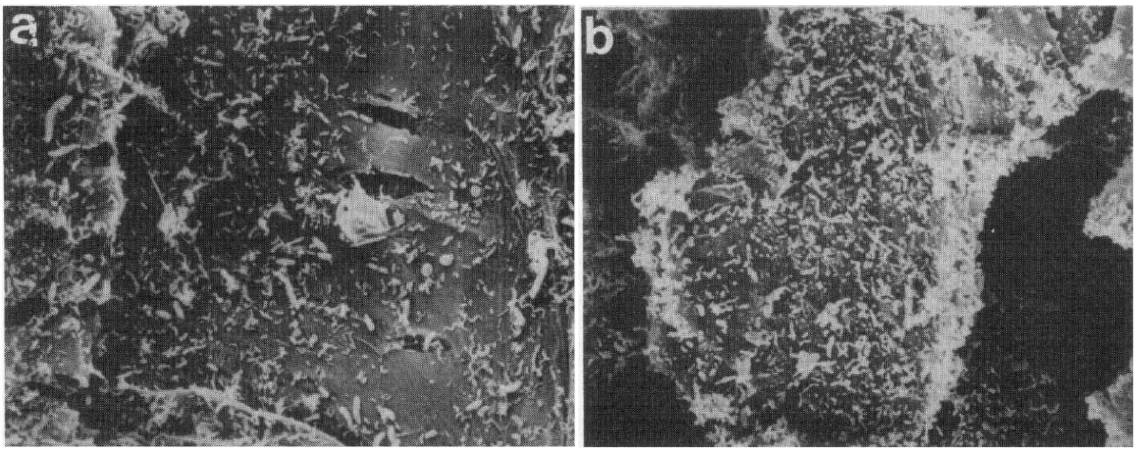

Figure 2.6: Scanning electron micrographs (×690 magnification) of lucerne stems after 6 hour in vitro incubation showing A) limited microbial adhesion to particle surface of normal stem and B) increased microbial adhesion to particle surface of shredded stem (Latham et al., 1978).

Feed intake

The quality of the forage affects the voluntary dry matter intake (**DMI**), which in turn affects the animal performance, and the feed intake is regulated due to various factors related to the physical limitations of the gastro-intestinal tract and to the metabolic status of the cow (Mertens, 1994) as shown in Figure 2.7. Feeding diets with high proportions of grass rather than concentrates, and especially grass harvested at late compared to early developmental stage, results in diets having high NDF concentration and diets that are bulky (Allen, 1996a). The slow digestion of rations high in NDF mostly results in selective retention of feed particles resulting in increased rumen pool sizes of NDF. The increased pool size of rumen content triggers neural stretch receptors in the rumen wall that signals the brain to cease a meal (Allen, 2000; Ingvartsen and Andersen, 2000). Different diets can result in equal rumen pool sizes of the NDF but might trigger very different levels of voluntary DMI, since the metabolic regulation can overrule the physical regulation of voluntary DMI depending on e.g. production level (Mertens, 1994). Moreover, the rumen pool size of NDF (measured as kg) is not the only factor affecting the receptors of the rumen wall, whereas e.g. increasing the volume rather than weight of the rumen content will elicit the stretching effect also (Allen, 1996a).

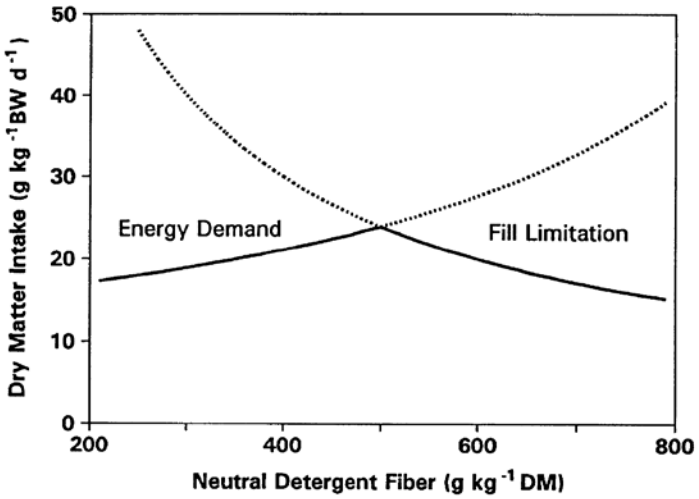

Figure 2.7: Effect of neutral detergent fibre concentration in feed on dry matter intake in dairy cows regulated by energy demand or fill limitations (Mertens, 1994)

A reduction in the forage particle size has shown to increase DMI by 0.5 kg/day for diets consisting of >50% forage, whereas NDF digestibility decreased 1.6 %-units (Nasrollahi et al., 2015). However, the meta-analysis by Nasrollahi et al. (2015) investigated the effects on both grass, lucerne, and maize, and due to lack of information or methods for suitable quantification of the particle size, the treatments were divided into the arbitrary groups of forage particle sizes (fine or coarse). Most studies comparing fresh and ensiled or dried forages have compared different crops. However, feeding silage instead of fresh crops or intensively dried crops (hay) reduces DMI in some cases, which is probably due to specific fermentation end products in the silage or the concentration of total acids in the silage (Huhtanen et al., 2007b; Grant and Ferraretto, 2018). Deficiencies in dietary CP might have a negative impact on DMI as undersupply of protein for the rumen microorganisms limits the microbial activity, resulting in less digestion and therefore reduced feed intake (Oldham, 1984). The increased DMI observed when increasing the concentration of CP up to 15% of DM in the diet is considered to be caused by the improved digestibility of DM, but the increased CP concentration is often mediated by increased concentrate proportion and thereby likely confounded with decreased NDF concentration (Oldham, 1984).

Digestion of protein

The concentration of CP in feedstuffs is determined as the concentration of N × 6.25, and CP can be grouped into either true protein or non-protein N (**NPN**). The true protein consists of molecular complexes made of amino acids (**AA**), whereas the rest, NPN, accounts for compounds such as urea, nitrate, ammonia-N, amines, amides, and free AA (McDonald et al., 2011). Moreover, CP is categorized as being either soluble or insoluble, which indirectly refers to the location of the CP, since most of the soluble CP is present in the leaves, whereas insoluble and fibre-bound protein is located mostly in the stem and cell walls (Solati et al., 2018). Dietary CP can be degraded in the rumen by microorganisms intracellularly, yielding mainly ammonia-N and VFA (Cotta and Russell, 1997) as shown in Figure 2.8. The degradability of CP in a given feedstuff can be determined in situ as the effective protein degradability (**EPD**), and the

CP, which is not degraded in the rumen, passes on through the omasum and abomasum to the small intestine for further digestion and is termed rumen undegraded protein (**RUP**).

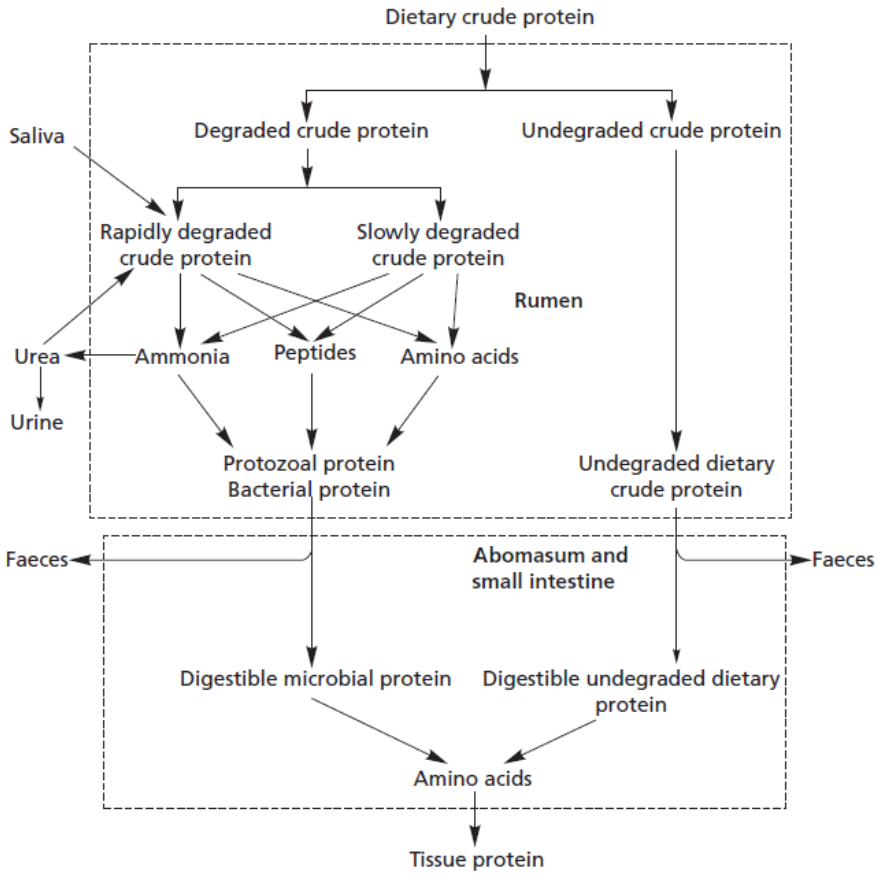

Figure 2.8: Schematic representation of degradation in the rumen and digestion in the small intestine of N-containing compounds (McDonald et al., 2011).

In ruminant nutrition, two types of protein requirements are assessed; that of the cow and that of the rumen microbes. The AAT/PBV-system quantifies the supply of AA in the small intestine (**AAT**) and the protein balance in the rumen (**PBV**) and is used in the Nordic feed evaluation system NorFor (Madsen et al., 1995; Volden, 2011). An AA can be categorised as essential, meaning the body is unable to synthesize it by itself, or non-essential, meaning the body can synthesise it (Boisen et al., 2000). However, rumen microbes can synthesise and supply the cow with essential AA, meaning that requirements for specific AA in the diet are lower compared to other animal species. However, high-yielding dairy cows benefit from an additional supply of rumen escape essential AA in the diet, indicating that the use of essential AA in ruminant nutrition is also of importance (Schwab and Broderick, 2017).

The PBV depends on rumen degradable protein from the feed and the energy supply and N availability for the microbial protein synthesis. The microbial protein synthesis is most often defined as the duodenal flow of microbial AA, since this is the amount of microbial AA that can potentially be digested and used by the cow (Clark et al., 1992; Lapierre et al., 2006). The efficiency of microbial protein synthesis depends on the substrates used for energy, since readily digested carbohydrates such as sugar and starch provide energy faster compared to

NDF, thereby increasing the microbial protein synthesis (Clark et al., 1992; Moorby et al., 2006). In addition to true protein supplied through feed, N for the microbial protein synthesis originates from NPN sources such as urea recycled from the body via saliva. The recycling of urea can help maintain microbial protein synthesis when amounts of rumen degradable CP in the feed is low (Reynolds and Kristensen, 2008).

The CP secreted or discarded from the body into the gastro-intestinal tract is defined as endogenous CP, and the duodenal flow of endogenous CP can be estimated as a fixed proportion of duodenal flow of DM (Larsen et al., 2000). In duodenum, flow of AA can have a microbial, dietary, or endogenous origin, and in most cases, microbial AA accounts for the majority of the duodenal flow of AA (Clark et al., 1992). The AA digested and absorbed in the small intestine is defined as the metabolisable protein (**MP**) and can be increased if the feed has a high proportion of RUP. However, during ensiling of grass, high amounts of true protein are converted into NPN, which is readily metabolised in the rumen (McDonald et al., 1991). Therefore, grass silage has a low proportion of RUP compared to fresh grass or intensively wilted grasses.

2.5 Production of methane and milk

Methane production

As described in previous sections of this chapter, rumen microbes ferment nutrients into mainly VFA (acetate, propionate, and butyrate) and other products. Microbes producing acetate and butyrate also produce CO_2 and H_2, whereas microbes producing propionate consume H_2 (Moss et al., 2000). The produced H_2 is readily absorbed in the rumen content, and the largest H_2 sink in the rumen is the methanogenic archaea, called methanogens, which use CO_2 and H_2 as substrates in the production of CH_4 (Moss et al., 2000). As indicated by stoichiometric calculations, the proportion of the three main VFA produced affects CH_4 production, since fermentation of 1 mole of glucose results in twice the amount of H_2 when acetate rather than butyrate is the end-product, whereas propionate formation consumes H_2.

Various mitigation strategies can be applied in the pursuit of decreasing the enteric CH_4 contribution from dairy cows (Boadi et al., 2004). When assessing the effects of e.g. dietary changes, the effect on the emission of CH_4 is normally expressed for CH_4 production (L/d), CH_4 yield (L/kg of DMI), or CH_4 intensity (L/kg of ECM). Improving the digestibility of grass would probably increase the CH_4 production due to higher DMI, but at the same time decrease the CH_4 yield (Boadi et al., 2004; Brask et al., 2013). Harvesting forages at an early compared to late developmental stage also reduces the NDF concentration of the forages, which thereby decreases the proportion of acetate in rumen fluid and consequently the CH_4 yield (Janssen, 2010). Shredding forage is another strategy towards increasing the digestibility of forage, which therefore has the potential to reduce the CH_4 yield. Pulp of e.g. grass has a higher NDF concentration compared to the parent material (Damborg et al., 2018), which suggests that CH_4 yield would be higher when feeding pulp compared to the whole plant forage (Brask et al., 2013). In contrast, digestibility is also expected to be higher in pulp compared to the whole plant forage, which would suggest a higher CH_4 yield when feeding whole plant forage compared to pulp. Studies investigating the difference in the emission of CH_4 when feeding

pulp and the whole plant forage is lacking, but a study in heifers showed increased CH_4 yield when feeding pulp (Hellwing et al., 2018).

As suggested above, altering the carbohydrate composition (i.e., the proportion of sugar, starch, and NDF) will affect the fermentation pattern in the rumen. It is well-known that increasing the proportion of concentrates in the diet might increase DMI and thereby the amount of OM available for fermentation (Moorby et al., 2006). The CH_4 production might therefore increase when increasing the concentrate proportion, but the CH_4 yield will decrease (Boadi et al., 2004) since the DMI generally increase relatively more than the CH_4 production (L/day).

Milk production

The animal performance of dairy cows (i.e., the milk production) depends mainly on intake of digestible nutrients and energy supply which is highly correlated to DMI (Mertens, 1994). The response in milk yield to increased intake of digestible OM is curve-linear, meaning that the marginal response in milk yield is positive but diminishing (Jensen et al., 2015; Daniel et al., 2016). To increase intake of digestible OM, concentrates are often supplemented, resulting in decreased NDF concentrations and higher concentrations of starch or CP in the diet. The response in milk yield diminishes since the marginal supply of energy decreases when the concentrate proportion increases (Coulon and Rémond, 1991). The response in milk production also diminishes due to a shift from the use of energy for milk production towards the deposition of energy in the body instead (Broster and Broster, 1984). Generally, increasing the intake of digestible OM also increases the yield of milk protein and milk fat and increases the milk protein concentration, whereas the milk fat concentration decreases (Larsen et al., 2013). The CP concentration of grasses is affected by e.g. season, which again affects the relative CP intake, especially if a nearly 100% forage diet is fed. The changes in CP concentration of the diet will affect mainly the milk yield, milk protein concentration, N efficiency (milk N/N intake), and milk urea concentration (Olmos Colmenero and Broderick, 2006). In the experiment by Olmos Colmenero and Broderick (2006), milk yield and milk protein yield did not increase when feeding diets higher than 16.5% CP of DM, whereas N efficiency decreased with increasing CP concentration in the diet. Further, Huhtanen and Hristov (2009) showed in a meta-analysis that CP concentration of the diet is the most important dietary factor influencing the N efficiency.

3 Aim and hypotheses

The objective of this PhD project was to investigate the effects of feeding grass harvested at different developmental stages as either fresh or as silage from physically processed grass on fibre digestibility, feed intake, milk production, and methane emission.

The following hypotheses are stated and assessed through experimental activities addressed in the following chapters:

A. Milk production in dairy cows increases when feeding fresh grass compared to ensiled grass.

B. Physical treatment of grass prior to ensiling increases fibre digestibility and feed intake.

C. Increased fibre digestibility in grass by early developmental stage and/or shredding reduces the enteric methane emission.

The hypotheses were tested in three feeding studies denoted the Fresh-Study, the Shred-Study, and the Pulp-Study, respectively. Hypothesis A was tested in the Fresh-Study, hypothesis B was tested in the Shred-Study and the Pulp-Study, and hypothesis C was tested in all three studies.

In Chapter 5, the results from the Fresh-Study are given in Paper I and II, the results from the Shred-Study are given in Paper III, and the results from the Pulp-Study are given in Paper IV and V.

4 Methodology

The thesis hypotheses were tested in three feeding studies. In the Fresh-Study, four dietary treatments were tested using 16 cows in their second lactation in a cross-over design with two periods. In the Shred-Study, four multi-fistulated primiparous cows were used in a 4 × 4 Latin square design with four periods of 21 days duration and a 2 × 2 factorial arrangement. In the Pulp-Study, six multiparous and multi-fistulated cows in mid to late-lactation were used in an incomplete 6 × 4 Latin square design with four periods of 21 days duration and a 2 × 3 factorial arrangement. This chapter includes a discussion of the incentives for the applied key methodological approaches towards answering the hypotheses. The specific methodological approaches for each experiment are described in Paper I-V in Chapter 5.

4.1 Study design

In vivo feeding studies and in situ studies were applied in this PhD project and the three feeding studies were designed as change-over experiments. Compared to a continuous design, each animal tests several treatments in a change-over design, and thereby acts as its own control, enabling adjustment of between animal variability. Consequently, a lower number of animals is required to test the treatment differences, which was of importance in the Shred-Study and Pulp-Study, where multi-fistulated animals were used. The change-over design was also chosen for the Fresh-Study, since the capacity for measuring gas exchanges using respiration chambers was limited to four cows at a time, thus limiting the number of animals that could be used in the experiment. Compared to the continuous design, the cross-over design might induce carry-over effects, meaning that the effect of a treatment in one period can affect the outcome of the treatment in the subsequent period. Inclusion of a sufficiently long adaptation period at the beginning of each experimental period ensured that the cow would be in a steady state regarding for example microbial population in the rumen, dry matter intake (**DMI**), marker flow, methane emission, and milk production. Because of the staggered approach resulting from limited capacity of respiration chambers for measurement of gas exchange, the adaptation period prior to digesta sampling in the Fresh-Study and the Pulp-Study ranged from 13 to 16 days and 11 to 13 days, respectively, whereas the adaptation period was 12 days for all cows in the Shred-Study. The chosen length of the adaptation period complied with the 7-14 days recommended by Grant et al. (2015).

The change-over design was chosen since it has been shown to be as accurate as and more precise than continuous designs in feeding trials using cows, as long as differences in DMI between experimental treatments do not exceed 5 kg/d (Huhtanen and Hetta, 2012). It was not expected that DMI would differ more than 5 kg/d when feeding shredded forage compared to a control forage (Wittenberg et al., 2000; Broderick et al., 2002) or pulp compared to the whole plant (Damborg et al., 2019; Savonen et al., 2019).

4.2 Digestibility and rumen kinetics

Digestibility is determined directly by measuring the intake and total faecal output of a given nutrient. The apparent total tract digestibility (**ATTD**) is calculated as:

$$\text{Apparent total tract digestibility} = \frac{[\text{Intake (kg/day)} - \text{Fecal output (kg/day)}]}{\text{Intake (kg/day)}}$$

Complications during total collection of faeces might arise, as separation of urine from faeces must be ensured, the amounts of faeces can be difficult to handle, and the consistency complicates quantitative sampling (Faichney, 1975, 1993). Moreover, the total collection procedure might affect the animal behaviour and thereby the DMI, and it can be costly due to the intensive labour required. As an alternative to total collection, representative spot-sampling of digesta and faeces can be utilised if flow markers are used (Ellis et al., 1994). Markers can be compounds or entities that exist in the feed naturally (internal marker) or they can be compounds that are supplemented to the diet or dosed into the animal (external marker). The perfect marker is defined as 1) unabsorbable, 2) not affecting or to affect by the gastrointestinal tract or its microbial population, 3) physically similar to or intimately associated with the material it is to mark, and 4) having a method of estimation that is specific and sensitive and the method must not interfere with other analyses (Faichney, 1975). However, the ideal marker does not exist (Faichney, 1993). In the included experiments, chromium oxide (Cr_2O_3) and titanium dioxide (TiO_2) were used as external markers. The Cr_2O_3 follows the flow of digesta and not any specific phases of the digesta (Titgemeyer, 1997), and TiO_2 behaves similar to Cr_2O_3 (Myers et al., 2006). The two markers were used to complement each other, but also to ensure that data quality could be maintained if problems with one marker arose.

In the Shred-Study and the Pulp-Study, cows were equipped with a cannula in duodenum and ileum to obtain spot-samples of digesta, since total collection at those sites is not tenable. Using the markers, the flow of nutrients could be determined at each site and used to quantify the apparent digestibility of each nutrient in the rumen, the small intestine, and the large intestine. The cannulas were simple T-shaped cannulas placed approximately 60 cm caudal to the pylorus and approximately 20 cm cranial to the caecum. In the Shred-Study and the Pulp-Study, the two markers were dosed into the rumen in connection with feeding twice daily to reduce the diurnal variation in marker flow, compared to only dosing once daily. The obtained samples should represent the diurnal variation in marker concentration in the digesta (Myers et al., 2006). Therefore, 12 spot samples were obtained during a 96 h period with on average 8 hours intervals in the Shred-Study and Pulp-Study, resulting in a representation of every second hour of the day in the pooled sample. The cows in the Fresh-Study were only supplied with TiO_2 as a marker, since they were in the food chain, where the milk is used for human consumption. The marker could not be mixed into the diet since cows were provided fresh grass, and it could not be provided in the concentrate supplementation, since only half of the dietary treatments included concentrates. Therefore, an additional pellet, containing minerals, TiO_2, wheat, rapeseed cake, and sugar beet molasses (to make it palatable), was produced and fed to the cows twice daily in connection with feeding. Compared to providing the marker on top of the forage, this approach ensured that all cows received the required amount of minerals and that all cows had a constant daily intake level of TiO_2. Over three days, faecal samples were collected twice daily and pooled to obtain a representative sample as done in previous literature (Johansen et al., 2017b).

As an alternative to the intensive in vivo studies, the digestibility of specific nutrients can be determined in situ by incubating samples of feed in the rumen of the cow. The method can be used for individual feedstuffs, whereas the digestibility obtained from the previously mentioned spot samples expresses the digestibility of the specific nutrient of all feedstuffs fed to the cow. The in situ method can be used to estimate the degradation rate of potentially degradable NDF. According to Norfor (Åkerlind et al., 2011), the procedure of the in situ study includes drying and milling of the sample prior to incubation, which partly simulates the effect of rumination including chewing by the cow, but also ensures that the low amount of material that is weighed out is representative. However, since it is hypothesised that physical processing of the forages will affect the fractional rate of digestion (**k_d**) of NDF, milling samples for the in situ study might mask the effect. Both in vivo and in situ studies were performed, although the results of the in situ study in the Pulp-Study are reported elsewhere (Bitsch, 2021). The k_d of digestible NDF (**DNDF**) and the fractional rate of passage of indigestible NDF (**iNDF**) can be estimated from rumen evacuations and estimations of flow of DNDF and iNDF. Despite that the in situ method for the determination of k_d of NDF is less resource demanding than determination by rumen evacuations, the method is still associated with some problems related to e.g. particle loss and restricted microbial activity (Mertens, 1993; Hvelplund and Weisbjerg, 2000).

4.3 Feeding management

In all three studies, cows were offered either forage as the sole feedstuff, forage with supplementation of concentrates divided into two daily offerings, or forage with supplementation of concentrates in a total mixed ration (**TMR**). Concentrates were either included or excluded in specific dietary treatments based on the research questions and practicalities. In the Fresh-Study and the Shred-Study, diets without concentrates were chosen to be representative for so-called low-input production systems, whereas including concentrates (approximately 6 kg/day or 35% of TMR on DM basis) in the Fresh-Study and the Pulp-Study represented practical feeding situations. Moreover, when assessing the effects of physical processing of a single feedstuff on digestibility of NDF, it is undesirable to dilute the obtained digesta samples from e.g. duodenum with NDF from other feedstuffs than the feedstuff that is actually tested. However, maintaining a high proportion of forage NDF in the total diet NDF can limit the errors associated with conclusions drawn on the effects of physical processing on e.g. ruminal NDF digestibility.

Fresh forage can be offered to the cow either by feeding harvested fresh forage in the barn (also termed zero-grazing) or by letting the animal graze. Despite grazing being common on organic farms due to legislations, the concept of zero-grazing was emphasised and since grazing and zero-grazing have resulted in different milk yields possibly caused by feed sorting and preferences (Mohammed et al., 2009), cows in the Fresh-Study were fed in the barn. Although methods do exist for the determination of DMI in grazing animals (Oudshoorn et al., 2013), direct measurement by subtracting left-overs from offered feed is considered accurate (Burns et al., 1994). Moreover, using respiration chambers is considered the golden standard method for determination of methane emission for dairy cows, although cows are more confined compared to other methods (Gardiner et al., 2015). The behaviour in DMI was considered to be less affected when moving the cows from individual stalls to the respiration chambers compared to introducing grazing cows to the respiration chambers.

5 Results

This chapter presents the results through papers and manuscripts derived from the three studies conducted in the PhD. The main results are stated here:

Paper I: *Effect of regrowth period for perennial ryegrass on yield and nutritive value of grass.* Prolonging the regrowth period resulted in increased growth rate and lower nutritive digestibility of the grass, but irrespective of the length of the regrowth period, large variation in both growth rate, chemical composition, and thereby nutritive value was observed through the 7-week study.

Paper II: *Effects on feed intake, milk production, and methane emission in dairy cows fed silage or fresh grass with concentrate or fresh grass harvested at early or late developmental stage without concentrate.* Feeding fresh perennial ryegrass instead of silage had no effect on DMI, or ECM yield, whereas increased proportions of C4-C16 fatty acids in milk suggested higher de novo synthesis in the utter of cows fed fresh grass. The DMI and ECM yield was higher in cows when concentrates were supplemented with fresh grass. Feeding cows fresh grass harvested at early compared to late developmental stage increased DMI and milk yield, but not ECM yield. The CH_4 yield (L/kg DMI) was lower, when concentrates were supplemented with fresh grass, but not different between cows fed silage and fresh grass or between cows fed fresh grass harvested at early and late developmental stages.

Paper III: *Shredding of grass-clover before ensiling: Effects on feed intake, digestibility, and methane production in dairy cows.* Feeding shredded compared to non-shredded (control) grass-clover silage to dairy cows in late lactation had no effect on DMI, ruminal digestibility of NDF, or milk yield, but decreased the ATTD of NDF and CH_4 production (L/kg of OM digested in the rumen). No benefits of shredding grass-clover harvested at late compared to early developmental stage were observed for digestibility measures, except for reduced time spent for chewing during rumination and total chewing.

Paper IV: *Effect of screw pressing and days of regrowth on grass silage characteristics and quality.* Pulp of perennial ryegrass pressed once or pressed twice during wet fractionation have different silage characteristics compared to the whole plant silage, but ensile just as well. Silage of pulp had a lower fermentation weight loss (g/kg fresh matter), higher density (kg DM/m^3), and lower concentrations of fermentation acids compared to whole plant silage. However, those parameters were confounded with silage DM concentration.

Paper V: *Fiber digestibility and protein value of pulp silage for lactating dairy cows – effects of wet fractionation by screw pressing of perennial ryegrass.* Feeding dairy cows with total mixed rations of concentrates and either early- or late-harvested grass silages of either chopped whole plant, pulp from one fractionation, or pulp from two fractionations resulted in interactions between developmental stage at harvest and type of processing. When processing grass harvested at late developmental stage, substituting the diet proportion of chopped whole plant grass silage with pulp pressed once, and then pulp pressed twice, respectively, ruminal digestibility of NDF increased linearly. When processing grass harvested at early developmental stage, substituting the diet proportion of chopped whole plant grass silage with pulp pressed once, and then pulp pressed twice, respectively, the protein value (g AA digested in the small intestine per kg DMI) increased linearly.

5.1 Paper I

Effect of regrowth period for perennial ryegrass on yield and nutritive value of grass

Hansen, N.P.,[1] T. Kristensen,[2] M. Johansen,[1] and M.R. Weisbjerg[1].

[1]Department of Animal Science, AU Foulum, Aarhus University, 8830 Tjele, Denmark
[2]Department of Agroecology, AU Foulum, Aarhus University, 8830 Tjele, Denmark

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Effect of regrowth period for perennial ryegrass on yield and nutritive value of grass

Hansen N.P.[1], Kristensen T.[2], Johansen M.[1] and Weisbjerg M.R.[1]
[1]Department of Animal Science, Aarhus University, Foulum, Denmark; [2]Department of Agroecology, Aarhus University, Foulum, Denmark

Abstract

During an eight-week period, perennial ryegrass was harvested at early and late maturity stage, corresponding to three and five weeks of regrowth. Each week, growth rate and stem proportion were determined, and chemical composition was analysed. During the experimental period, longer regrowth period resulted in increased growth rate and a lower nutritive quality of the grass. Irrespective of regrowth period, there was a large variation in growth rate and chemical composition, and thereby also in nutritive quality throughout the experiment. When optimising cutting strategy, these relations should be assessed in conjunction with the effects on feed intake and milk performance when grass is fed to dairy cows.

Keywords: perennial ryegrass, growth rate, organic matter digestibility, stem proportion

Introduction

Barn feeding with fresh cut grass has gained increasing interest for dairy cow management in Denmark. However, both the quality and quantity of grass change throughout the growing season and knowledge about field yield and nutritive value of the grass is a prerequisite for the farmer to optimise both grassland yields and utilisation of the grass by the cows. With longer regrowth period, field yield might increase, but digestibility of organic matter will decrease and dairy cows might respond by reducing feed intake (Johansen *et al.*, 2017). The objective of this experiment was to study the effects of a constant regrowth period of perennial ryegrass on growth characteristics and chemical composition. The presented results were obtained during a larger study, where the effects of harvesting grass with different lengths of period for regrowth on feed intake and milk performance in dairy cows were investigated also, but these results are not included in this paper.

Materials and methods

An experiment was conducted from mid-May to start-July 2019 at Aarhus University, Foulum, Denmark. The experiment started in week 20 and lasted for 8 weeks. In the experiment, fresh grass was harvested at early (3 weeks of regrowth; EG) and late (5 weeks of regrowth; LG) maturity stage. A field of perennial ryegrass (*Lolium perenne*) was divided into two blocks that were further divided into three and five plots for EG and LG, respectively. During the experiment, one plot from each of EG and LG were harvested at a time over a one-week period in order to supply feed for the feeding trial. By the end of the week, the remaining grass in the respective plots was harvested and removed. Each day, weight and dry matter (DM) content of harvested grass was measured to determine DM yield, and the sum within plot was used to calculate average growth rate during the 3 or 5 weeks of growth for EG and LG, respectively. All plots were fertilised with 78 kg N ha^{-1} on 2 March, and 78 kg N ha^{-1} on 28 May (week 22) or 4 June (the two plots that were harvested during week 22). Before the experiment started, plots within EG and LG were managed to obtain three and five weeks of regrowth. For EG, grass from all three plots were harvested and removed three weeks prior to harvest for each plot (Monday week 17, 18, and 19, respectively). For LG, grass in the first plot remained untouched, grass in the second plot was mown (Monday week 16), and grass in the remaining three plots were harvested and removed five weeks prior to harvest for each plot (Monday week 17, 18, and 19, respectively). After all plots had been harvested the first time during the experiment (starting from week 20), plots were harvested a second time in the same order, and for

EG, two plots were harvested a third time (week 26 and 27). The grass was harvested with a direct cut and loader wagon (Grass Tech Grazer, Borris, Co. Carlow, Ireland). Thursday each week, a subsample (approx. 130 g) of EG and LG, respectively, was divided into leaves (leaf blade) and stems (leave sheath, stem, and flower) by hand prior to DM determination, to determine leaf-to-stem ratio on DM basis. Within EG and LG, representative samples from harvested grass were pooled across three days weekly from week 22 to 27 for chemical analysis of neutral detergent fibre (NDF), acid detergent fibre (ADF), *in vitro* organic matter (OM) digestibility, and crude protein (CP). Statistical analyses were done in R (version 3.5.2) and the following model was used to analyse data for stem proportion and chemical composition:

$$Y_{mw} = \mu + \alpha_m + \beta_w + E_{mw}$$

where Y is the dependent variable, μ is the overall mean, α is the fixed effect of maturity stage (m = EG, LG), β is the fixed effect of week (w = 20 to 27 for stem proportion and w = 22 to 27 for chemical composition), and E is the random residual error assumed to be independent and normal distributed.

Results and discussion

Growth rate (kg DM ha^{-1} day^{-1}) varied throughout the experiment for both EG and LG (Figure 1A). EG was almost equal to LG in week 20 and 21, probably due to a very cold period in the beginning of May. In addition, grass harvested in week 21 had been mown five weeks earlier during cold weather, possibly hampering the regrowth in the beginning of that period. A limited amount of rain was recorded until week 24, when 34% of the total rainfall during the experimental period was recorded. The combination of rainfall and an increasing temperature (mean daily temperature exceeded 15 °C during week 23) probably caused the increased growth rate (Buxton, 1996) for week 24. As the temperature increased, the stem proportion and the concentration of NDF and ADF also increased (Figure 1B, C, and D, respectively) for both EG and LG. Across all weeks, stem proportion, NDF and ADF concentration were 14.4%-units, 46 and 34 g kg DM^{-1} higher in LG than EG (P<0.01). Stem proportion peaked in week 23 and 24 for EG and LG, respectively. The subsequent decrease in stem proportion in weeks corresponding to the second cut reflected that stem proportion in the second cut in general decreases when the first cut is delayed (Soegaard *et al.*, 2011). The change in stem proportion was reflected in the concentration of NDF and ADF, which peaked in week 24 and declined for the rest of the experiment. The increase in stem proportion is well reflected in a decreasing OM digestibility (Figure 1E), whereas OM digestibility starts to increase as stem proportion decreases. Across all weeks, OM digestibility was 3.6%-units higher in EG compared to LG (P<0.01). The difference in CP concentration between EG and LG was relatively constant from week 22 to 25 (Figure 1F). Until week 25, all plots had been fertilised twice; 50% before the experiment started and 50% after and during the first harvest for EG and LG, respectively. In week 26 and 27, grass from the plots in EG was harvested for the third time without having received further N fertiliser after the second harvest. The low concentration of CP in EG during week 26 and 27 might therefore have been caused by a depletion of N in the soil.

Conclusions

Longer regrowth period resulted in increased growth rate but lower nutritive quality of perennial ryegrass from mid-May until the beginning of July. Generally, there was large variation over time irrespective of regrowth period. The variation was related to weather conditions and nutrient accessibility. Difference on DM yield and nutritive quality will be assessed in conjunction with the effects on feed intake and milk performance, when subsequently feeding the grass to dairy cows.

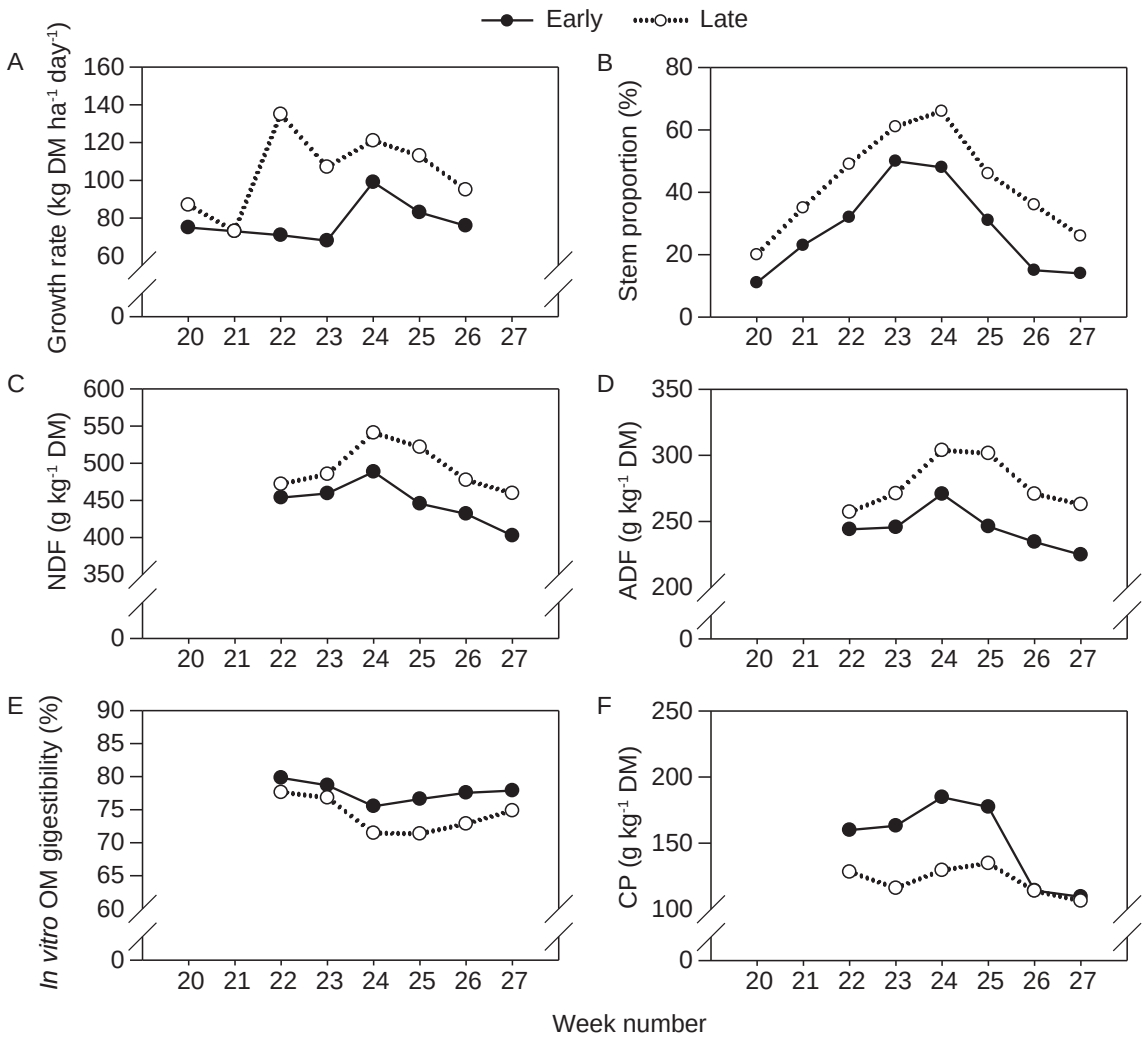

Figure 1. Development in growth rate (A), stem proportion (B), neutral detergent fibre (NDF) concentration (C), acid detergent fibre (ADF) concentration (D), *in vitro* organic matter (OM) digestibility (E), and crude protein (CP) concentration (F) in early (3 weeks regrowth) and late (5 weeks regrowth) cut grass during the experiment.

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5.2 Paper II

Effects on feed intake, milk production and methane emission in dairy cows fed silage or fresh grass harvested at early or late development stage with and without concentrate

Hansen, N.P.,[1] T. Kristensen,[2] M. Johansen,[1] L. Wiking,[3] N.A. Poulsen,[3] A.L.F. Hellwing, [1] L. Foldager,[1] S.K. Jensen,[1] L.B. Larsen,[3] and M.R. Weisbjerg[1].

[1]Department of Animal Science, AU Foulum, Aarhus University, 8830 Tjele, Denmark
[2]Department of Agroecology, AU Foulum, Aarhus University, 8830 Tjele, Denmark
[3]Department of Food Science, Aarhus University, 8200 Aarhus N, Denmark

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Interpretive Summary

Effects on feed intake, milk production, and methane emission in dairy cows fed silage or fresh grass with concentrate or fresh grass harvested at early or late development stage without concentrate.

Hansen et al.

Inclusion of grass in crop rotations have positive environmental effects. Approaches to increase intake of grass in dairy cows are therefore needed, and this study shows that utilization method, concentrate supplementation, and harvest strategy of grass in the field affect feed intake and the succeeding response in milk and methane production.

FRESH GRASS FOR DAIRY COWS

Effects on feed intake, milk production, and methane emission in dairy cows fed silage or fresh grass with concentrate or fresh grass harvested at early or late development stage without concentrate

N. P. Hansen,[1] T. Kristensen,[2] M. Johansen,[1] L. Wiking,[3] N. A. Poulsen,[3] A. L. F. Hellwing,[1] L. Foldager,[1,4] S. K. Jensen,[1] L. B. Larsen,[3] and M. R. Weisbjerg[1]

[1]Department of Animal Science, AU Foulum, Aarhus University, 8830 Tjele, Denmark

[2]Department of Agroecology, AU Foulum, Aarhus University, 8830 Tjele, Denmark

[3]Department of Food Science, Aarhus University, 8200 Aarhus N, Denmark

[4]Bioinformatics Research Centre, Aarhus University, 8000 Aarhus C, Denmark

Corresponding author: Nikolaj Peder Hansen, Blichers Allé 20, 8830 Tjele, Denmark. Phone: +45 28605590. E-mail: nikolaj.hansen@anis.au.dk

ABSTRACT

The objective of the study was to quantify the effects on dry matter intake (DMI), nutrient digestibility, gas exchange, milk production, and milk quality in dairy cows fed fresh grass harvested at different developmental stages. Sixteen Danish Holstein cows in mid-lactation were used in a cross-over design with two periods of 21 days. The cows received one of four treatments in each period: grass-clover silage supplemented with 6 kg/d of concentrate (SILc), fresh grass harvested at late developmental stage supplemented with 6 kg/d of concentrate pellets (LATc), fresh grass harvested at late developmental stage (LAT), and fresh grass harvested at early developmental stage (ERL). The cows were housed in tie-stalls and milked twice daily. The cows had ad libitum access to the forage and each type of pellet was divided into equal amounts and fed separately in the morning and afternoon. At the end of each period, fecal samples were collected to determine apparent total tract digestibility (ATTD), and samples of rumen fluid were collected with an oral flora sampler for determination of rumen short chain fatty acid composition. Halters were used for measuring eating and rumination time. In addition, cows were moved to open-circuit respiration chambers for gas exchange measurements. The ATTD of organic matter (OM) was lower for LATc compared to SILc, whereas DMI and energy corrected milk (ECM) yield did not differ between the two despite numeric differences. The fatty acid (FA) proportions of ∑C4-C10, ∑C12-C14, and ∑C16 in milk were higher for SILc compared to LATc, signifying pronounced de novo synthesis. The n6:n3 ratio in milk was lower for SILc and LAT compared to LATc, indicating improved nutritional quality for SILc and LAT. However, retinol concentration in milk was lower in SILc compared to all other treatments. Unexpectedly, CH_4 yield (L/kg of DMI) was not different between SILc and LATc, where relatively large differences in concentrations of water soluble carbohydrates were observed between forages. Relative to SILc, cows fed fresh grass experienced a convex pattern in DMI during the experiment. The changes in DMI were related to changes in stem proportion, fiber concentration, and OM digestibility determined in vitro in samples of the fresh

grass harvested throughout the experiment. Compared to LAT, LATc had higher DMI, ECM yield, milk protein yield, and chewing time, whereas milk fat concentration and ATTD of OM were lower. The CH_4 yield was higher for LAT compared to LATc, but was also unrelated to short chain FA composition in rumen liquid. The ATTD of OM was not different between LAT and ERL, possibly caused by a higher DMI for ERL. Milk yield but not ECM yield was higher for ERL compared to LAT, and unexpectedly, there was no difference in CH_4 yield or intensity between the two. The study implies that managing a grass-based diet in relation to utilization method and concentrate supplementation can influence both milk production, milk quality, and its impact on climate and environment.

Key words: Casein, fatty acid, perennial ryegrass, ruminant, zero-grazing.

INTRODUCTION

Dairy production based on mainly grass either as silage, grazed, or harvested grass fed in the barn, is extensively used in regions with temperate climates such as in Europe, United States, and New Zealand (Moscovici Joubran et al., 2021). Grass-based production systems accommodate both consumer trends towards greater willingness to pay a premium for pasture-raised (i.e. organic) products from agriculture (Stampa et al., 2020) and crop production with high carbon sequestration. There are advantages and disadvantages linked to grazing rather than delivering freshly harvested grass to the cows in the barn. Increasing herd sizes on organic farms advocates for rethinking grazing managements to overcome high grazing pressure due to increased stocking rate, and to avoid long walking distances to grasslands in the distant periphery of the farm (Van den Pol-van Dasselaar et al., 2008).

During the ensiling process, microbial activity causes protein to hydrolyze and deaminate, and sugars are fermented resulting in less potential substrate for microbial protein synthesis in the

rumen. Therefore, grass silage is attributed with a low concentration of MP (Johansen et al., 2017), especially compared to barn-fed fresh grass, where no fermentation and only limited proteolysis has occurred in the crop during harvest and feeding (Slottner and Bertilsson, 2006). Feeding of fresh grass rather than grass silage has shown potential for increasing milk production in terms of increased supply of MP (Younge et al., 2004), which is a major factor affecting milk production. Concentrate supplementation can substantially increase milk production, but may also give rise to lower digestibility and thereby lower nutrient utilization due to higher rates of passage (Krizsan et al., 2010).

Compared to concentrate-based feeding, pasture-based feeding has shown to affect coagulation properties of milk; however, the mechanisms seems equivocal (Magan et al., 2021). Despite some studies have reported effects of dietary alterations on vitamin concentrations in milk (Adler et al., 2013; Poulsen et al., 2015), knowledge on dietary changes in pure grass-based diets with and without concentrates is still limited. Moreover, feeding fresh grass rather than silage has shown to affect milk fatty acid (**FA**) composition and the proportion of e.g. PUFA increases (Elgersma, 2015). The concept of grass milk, i.e. nearly 100% forage-based diet, has further shown to reduce the n6:n3 FA ratio in milk (Benbrook et al., 2018), thereby improving the nutritive value of milk for human nutrition.

More sugar in fresh compared to ensiled grass will likely be available for microbial fermentation in the rumen, suggesting higher CH_4 yield (L/kg of DMI), when feeding fresh grass (Børsting et al., 2020). Furthermore, improving the digestibility of silage in order to increase milk production has been suggested as a mitigation strategy (Brask et al., 2013) as CH_4 yield is lower in cows fed grass silage harvested at early compared to late developmental stage. Similar trends in CH_4 yield may also be attained, when feeding fresh grass harvested at early compared to late developmental stage.

The aim of the current study was to investigate how managing a grass-based diet in relation to utilization method and concentration supplementation for dairy cows affected DMI, milk yield, milk quality, and production of CH_4. We hypothesize 1) that feeding of fresh grass compared to silage at comparable OM digestibility (**OMD**) will result in higher DMI and ECM yield and improve the milk quality in relation to protein and FA composition, 2) that supplementation of concentrate while feeding fresh grass will increase milk production, and 3) that feeding of fresh grass harvested at early compared to late developmental stage will increase milk production and decrease CH_4 yield.

MATERIALS AND METHODS

The experiment complied with the guidelines set out by the Danish Ministry of Environment and Food Law No. 474 (May 15, 2014) concerning animal experiments and care of animals used for scientific purposes.

Forage production

The production and harvest of fresh grass in the current experiment have previously been reported in Hansen et al. (2020). In spring 2018, a field of perennial ryegrass (*Lolium perenne* L., a mix of the varieties ‘Ovambo 1’, ‘Bovini’, and ‘Masai’) and white clover (*Trifolium repens* L., a mix of the varieties ‘Silvester’ and ‘Rivendel’) was established near Tjele, Denmark (56.49° N 9.60° E) with barley as cover crop. During autumn 2018, the grass was harvested and removed from the field once. Clover (determined in botanical analyses during harvest for the experiment) constituted less than 1% of the forage on DM-basis, and the crop was therefore defined as only grass. In 2019, the fresh grass used for the feeding experiment was harvested at early or late developmental stage, corresponding to a 21 and 35 d period of regrowth, respectively. The fresh grass was harvested daily and used for feeding in the barn from Monday on May 13 (start of experimental week 0) to July 3 (week 7). To ensure a constant difference in developmental stage,

the field was divided into two plots, which were further divided into three and five subplots, from which grass was harvested at an early and late developmental stage, respectively. During the feeding experiment, fresh grass of each developmental stage was harvested each from one subplot at a time for a one-week period. After all subplots were harvested once, starting in week 0, harvest proceeded on the subplots a second time in the same order for grass of both developmental stages, and a third time for two subplots of grass of early developmental stage (week 6 and 7). By the end of each week, remaining sward in the subplots was harvested and removed from the field. To obtain three and five weeks of regrowth during harvest in the first weeks of the feeding experiment, grass in subplots of the early developmental stage was harvested and removed three weeks before the first harvest of the respective subplot during the feeding experiment, corresponding to week -3, -2, and -1. For subplots of grass of late developmental stage, grass in the first subplot was untouched (week -5), and grass in the remaining four subplots were harvested and removed five weeks prior to harvest of each of the subplots corresponding to week -4, -3, -2, and -1. All subplots were fertilized with 78 kg N per ha on March 2 (week -11) as well as 78 kg N per ha on May 28 (week 2) or June 4 (week 3; the two subplots harvested during week 2). The fresh grass was harvested and loaded directly once daily between 1000 and 1100 h using a Grazer GT120B wagon (Grass Technology Ltd.). Samples of harvested grass of each developmental stage were collected Thursday in week 0-7 for determination of stem proportion on DM-basis, where approx. 130 g of grass was divided into leaves and stems (leave sheath, stem, and flower) prior to DM determination (60°C in air-forced oven for 48 h). In week 0-7, developmental stage was determined in the field for the subplot being harvested according to Moore et al. (1991).

The forage used for grass-clover silage was composed of perennial ryegrass (*Lolium perenne* L., a mix of the varieties ‘Abosan 1’ and ‘Saqui’), hybrid ryegrass (*Lolium*×*boucheanum* ‘Tetratop’), white clover (*Trifolium repens* L. ‘Silvester’), and red clover (*Trifolium pratense* L. ‘Callisto’). The grass-clover was cut, wilted, precision chopped, and ensiled in primary growth in

the spring of 2018, aiming at same developmental stage as fresh grass harvested at the late developmental stage.

Animals and housing

Four dietary treatments were tested using 16 second lactation Danish Holstein cows in a cross-over design with two experimental periods, each of 21 d duration. The cows were housed in a tie-stall in cubicles with rubber mats and sawdust as bedding material and free access to water. At the beginning of the experiment, the cows averaged (mean ± SD): milk yield, 31.6 ± 5.4 kg/d; DMI, 19.3 ± 2.5 kg/d; DIM, 153 ± 48 d. The cows were blocked according to DIM in blocks of four cows, and within each block, cows were randomly assigned to treatments. The blocks were balanced for carry-over effects. The dietary treatments were grass-clover silage supplemented with 6 kg/d of concentrate pellets (**SILc**), fresh grass cut at a late developmental stage supplemented with 6 kg/d of concentrate pellets (**LATc**), fresh grass cut at a late developmental stage (**LAT**), and fresh grass cut at an early developmental stage (**ERL**). In addition, all cows received 600 g/d of marker pellets, which beside the same ingredients as the concentrate pellets consisted of minerals (VM2 grøn; Vilofoss), vitamins (Supplex ADE, Vilofoss), and titanium(IV) dioxide as an external digesta flow marker (Table 1). The cows were fed twice daily at 0600 and 1545 h, where forage was offered for ad libitum intake, and concentrate and marker pellets divided equal for each feeding were offered in a separate trough associated to each cubicle. The forage refusals were removed and weighted before the afternoon feeding, and the amount of new forage offered were adjusted aiming at 15% refusals (DM-basis). The cows were milked twice daily at 0515 and 1530 h.

The experimental periods for the four blocks of cows started in a staggered order, since the capacity for measuring gas production using respiration chambers was limited to one block (four cows) at the time (described below). Therefore, period 1 for block 1 and 2 started Monday and

Thursday in week 0, respectively, whereas period 1 for block 3 and 4 started Monday and Thursday in week 1, respectively.

Sampling and recording

Samples of silage and fresh grass were, in each experimental period, collected on d 16 to 18 for block 1 and 3, on d 13 to 15 for block 2 and 4 (during digestibility measurement, equal Tuesday to Thursday) and on d 19 to 21 for all blocks (during CH_4 measurement), and feed residues were sampled the following day. The forage residues and a subsample of each forage type were used for determination of DM (air-forced oven at 60°C for 48 h). The remaining sample fraction of each forage type from each of those days was stored at -20°C until the end of the experiment. After thawing, those samples were pooled across three consecutive days, representing time points, where DMI was measured. Concentrate and marker pellets were collected once weekly (Tuesday) throughout the experiment and stored at -20°C. At the end of the experiment, thawed samples of the pellets were pooled within pellet type and experimental period and half of the pooled samples were used for determination of DM concentration (60°C in air-forced oven for 48 h), while the other half was stored at -20°C until chemical analysis.

The time cows spent chewing during eating and rumination was measured using the RumiWatch noseband sensor (ITIN+HOCH GmbH) from d 15 to 19 for block 1 and 3 and from d 12 to 16 for block 2 and 4 (equal Monday to Friday). As recommended by Zehner et al. (2017), the raw data was converted using RumiWatch Converter version 0.7.3.2 (ITIN+HOCH GmbH), and output data was selected for a one-hour resolution.

In each experimental period, six samples of feces (350 mL) were collected when cows defecated or after stimulation at 0800 and 1400 h on d 17 to 19 for block 1 and 3, and on d 14 to 16 for block 2 and 4 (equal Wednesday morning to Friday afternoon). The samples were stored at -20°C and pooled within cow and experimental period. After the experiment, samples were

thawed and dried in an air-forced oven at 60°C for 48 h to determine the DM concentration, and subsequently stored at room temperature until chemical analysis.

As described by Larsen et al. (2020), samples of rumen fluid (25 mL per cow) were obtained using the ororuminal FLORA sampling device (Profs Products) at 1130 h on d 19 for block 1 and 3 and on d 16 for block 2 and 4 (equal Friday). Samples were stabilized using 25% meta-phosphoric acid in the ratio 1:4 and stored at -20°C until chemical analysis.

On d 19 in each experimental period, cows were moved to individual open-circuit respiration chambers (17 m^3) built of a steel frame with transparent polycarbonate walls (Hellwing et al., 2012). The chambers were placed in a square, where the cows faced each other, and the individual cow was placed in the same chamber in both experimental period 1 and 2. The cows were milked and fed twice daily starting at 0515 and 1530 h. One cow was milked and fed at a time (required approximately 15 min) before the next chamber was opened. For 30 min after closing the chamber, cows were restricted access to the feed until a lid covering the feed was automatically removed. For three days starting on d 19 for all blocks, the concentrations of CH_4, CO_2, H_2, and O_2 were measured in the inlet air (background air), and in the air coming from each chamber. The concentrations were measured every 12.5 min. The airflow was measured using a HFM-200 flow meter with a laminar flow element (Teledyne Hastings Instruments), the CH_4 concentration was measured using an infrared sensor (VIA-510, Horiba Instruments), the H_2 concentration using an electrochemical sensor (3HYT CiTiceL, Honeywell International Inc.), the CO_2 concentration using an infrared sensor, and O_2 concentration using a paramagnetic sensor, both from Columbus Instruments International. On d 19 in each experimental period, the gas sensors were calibrated using dinitrogen (N_2) for the zero point calibration and a span gas (20.5% O_2, 5000 ppm CO_2, 500 ppm CH_4, 150 ppm H_2, and N_2 as the remainder part; AGA A/S) for the top point calibration. See Hellwing et al. (2012) for further details.

Data for gas measurements obtained while chamber doors were opened were deleted. The 24 h gas production was calculated as the accumulated gas production during the total measurement period divided by the total measuring time (min) multiplied with 1440 min. Furthermore, the yield of gasses in each chamber were corrected individually for the gas recovery determined at recovery tests performed before, during, and after the feeding experiment. A total of 52 CO_2 recovery tests with an average recovery (± SD) of 99.6 ± 1.2% and a total of 25 CH_4 recovery tests with an average recovery of 99.6 ± 2.5% were performed. The recovery of O_2 and H_2 was calculated as the average of the recovery of CO_2 and CH_4. The reported gas production is given in L under standard conditions (0°C, 101.325 kPa). The CH_4 produce was expressed as production (L/d), yield (L/kg of DMI), and intensity (L/kg of ECM).

Milk yield was measured for six consecutive milkings on d 16 to 18 for block 1 and 3, on d 13 to 15 for block 2 and 4 (during digestibility measurements, Tuesday afternoon until Friday morning), and on d 19 to 21 for all blocks (during gas measurements). The main milk samples were collected during four consecutive milkings in each milk measurement period starting at the third milking and analyzed for overall composition. Furthermore, additional milk samples were obtained during two consecutive milkings on d 16 for block 1 and 3 and on d 13 for block 2 and 4 (Tuesday during digestibility measurement). The additional milk samples were analyzed for FA and protein composition, concentration of minor constituents, and coagulation properties (described below).

Feed and feces analysis

Samples of silage, fresh grass, concentrate pellets, and marker pellets were freeze-dried and then, as for samples of feces, milled through a 1 mm screen (ZM 200 mill, Retch GmbH) prior to chemical analysis. Ash was determined by combusting at 525°C for 6 h. Titanium(IV) dioxide was determined using spectrophotometry after digestion with H_2SO_4 followed by addition of H_2O_2 as described by Myers et al. (2004) with the modification that 15 instead of 10 mL of 30% H_2O_2 were

added and an additional five drops of H_2O_2 were added prior to measuring the absorbance. In samples of silage, fresh grass, concentrate pellets, and marker pellets, FA composition was quantified using GC (Agilent technologies) after adding pure C19:0 FA (Sigma-Aldrich Chemie GmbH) as internal standard (Jensen, 2008). Total N was analyzed using the Dumas principle (Hansen, 1989) in a Vario Max CN (Elementar Analysesysteme GmbH) and CP was calculated as total N × 6.25. Soluble N was determined by extraction in a borate-phosphate buffer (pH 6.75) at 39°C for 1 h (Åkerlind et al., 2011). The concentration of NDF, ADF, and ADL was determined sequentially using an ANKOM 220 Fiber Analyzer (ANKOM Technology). In the procedure, heat-stable α-amylase and Na_2SO_3 were used (Mertens, 2002) and ash correction was performed with the ash residues remaining in the ADL residue. In silage and fresh grass, the in vitro OM digestibility was determined by incubating samples in rumen fluid for 48 h and subsequently incubating in an HCl and pepsin solution for 48 h (Tilley and Terry, 1963). For concentrate and marker pellets, enzymatic OM digestibility was determined by treating samples with a solution of HCl and pepsin, and subsequently incubating samples in a solution of cellulolytic enzymes (Álvarez et al., 2020). The in vivo OMD was then calculated for forage as 4.10 + 0.959 × in vitro OMD and for pellets as 5.38 + 0.867 × enzymatic OM digestibility as described in Åkerlind et al. (2011)

As outlined by Larsson and Bengtsson (1983), milled samples (0.5 mm; Tube Mill control, IKA-Werke BmbH & CO. KG) of fresh grass and silages were analyzed for glucose, fructose, sucrose, and fructans using an enzymatic colorimetric method after extraction with a 0.1 M acetate buffer. Total water soluble carbohydrates (**WSC**) were defined as the sum of the four analytes.

The concentration of indigestible NDF (**iNDF**) was determined in freeze-dried and milled (1.5 mm; Pulverisette 15, Fritsch GmbH) samples of silage, fresh grass, concentrate pellets, and marker pellets as described in Åkerlind et al. (2011). In short, three non-lactating and rumen cannulated (#1C, Bar Diamond Inc.) cows were fed at maintenance level with a ration having a 69:31 forage to concentrate ratio (hay as primary forage and barley and oat grain as primary concentrate). Samples

weighed out in Dacron bags (2 g per bag; 12 µm pore size; Saatitech S.p.A.) were incubated in the rumen of the three cows (three replicates; one bag per cow per sample) for 288 h. The NDF residue (ash free) remaining in the bag (equivalent to iNDF) was analyzed using a Fibertech M6 System (Foss Analytical).

Extracts of silage were produced as described by Johansen et al. (2017), except that 25% meta-phosphoric acid was used for stabilization after pH was measured. Rumen fluid and silage extracts were analyzed for VFA using GC (Kristensen et al., 1996). In silage extracts, ammonia N was analyzed using a Cobas Mira auto-analyzer (Triolab A/S) after diluting samples (1:20) in a phosphate buffer (AM 1015, Randox Laboratories Ltd.), and L-lactate was quantified using the immobilized oxidase electrode technique (Mason, 1983; YSI 2900D, YSI Inc.). The sum of L-lactate and the analyzed individual VFA constituted the short-chain FA (**SCFA**).

Milk analysis

The main milk samples were analyzed for overall composition of fat, protein, and lactose monohydrate using a Milkoscan 4000 analyzer (Foss Analytical) at Eurofins Steins. In additional milk samples, urea and citrate were measured on full milk by infrared spectroscopy (Milkoscan FT2, Foss Analytical). The instrument is routinely calibrated for these parameters. Total calcium was measured by potentiometric titration as outlined by Poulsen et al. (2017). After skimming (centrifugation for 30 min, 2,643 × *g* at 4°C), pH (PHM220 pH meter, Radiometer Analytical) and conductivity (CDM210 conductivity meter, Radiometer Analytical) were measured. Furthermore, coagulation properties were measured on fresh skim milk samples as outlined by Frederiksen et al. (2011). Protein profiling was performed as described by Jensen et al. (2012). Milk FA were quantified using GC as described by Larsen et al. (2013), except that heptane instead of pentane was used as solvent. Retinol and α-tocopherol were quantified using HPLC after saponification and

297 extraction into heptane as described by Jensen (1994) and Jensen et al. (1998), respectively.
298 Riboflavin was quantified in skim milk using RP-HPLC as described by Poulsen et al. (2015).
299
300 ***Calculations and Statistical Analysis***
301 The concentration of net energy for lactation (**NEL**), AA absorbed in the small intestine
302 (**AAT**), and the protein balance in the rumen (**PBV**) were determined in NorFor (Volden, 2011)
303 using means of the chemical composition of each forage type, concentrate pellet, and marker pellet
304 (Table 1). Intake of DM and milk yield per day were averaged within cow, experimental period, and
305 time within experimental period (during digestibility measurement or during gas measurement).
306 Milk fat, protein, and lactose were calculated as the yield-weighted average in the samples collected
307 during the four consecutive milkings. Energy corrected milk yield (3.14 MJ/kg) was calculated
308 using the formula (Sjaunja et al., 1990): ECM yield (kg/d) = milk yield (kg) × [(38.3 × fat (g/kg) +
309 24.2 × protein (g/kg) + 15.71 × lactose (g/kg) + 20.7) / 3,140], where lactose is lactose
310 monohydrate. The feed conversion ratio was calculated as: ECM yield (kg/d) / DMI (kg/d), and the
311 N use efficiency (**NUE**) as: [Milk protein (kg/d) / 6.38] / N intake (kg/d) × 100. Fecal output of DM
312 was determined using titanium(IV) dioxide as marker, and then used for calculating the fecal flow
313 of OM and subsequently, the apparent total tract digestibility (**ATTD**) of DM and OM. For
314 analyzed milk components measured in the additional milk samples, yield-weighted averages were
315 calculated per cow per experimental period. The production of CH_4 was expressed in relation to
316 DMI and ECM yield (CH_4 yield and intensity, respectively), both determined during the gas
317 measurement period.
318 The statistical analyses were performed using R 4.0.4 (R Core Team, 2021), where the effect of
319 dietary treatments were analyzed using a linear mixed effects model with the '*lmer*' function from
320 the '*lme4*' package (Bates et al., 2015). The data was divided into three datasets as described below.

Dataset 1 included the response variables for feed intake, milk production, feed efficiency, and NUE (Table 3) measured twice within each experimental period (both during digestibility measurement and during CH_4 measurement), and variables were analyzed using the model described by Equation 1. The model used was a regression model based on weeks in the experiment, where experimental period is embedded in the variable week (w).

$$Y_{ijk} = \alpha_i + \beta \times 1_{\{Grass\}} \times w_{ijk} + \gamma \times 1_{\{Grass\}} \times w_{ijk}^2 + \upsilon_j + \varepsilon_{ijk} \quad (1)$$

For the model in Equation 1, Y_{ijk} is the dependent response variable, α_i is the effect of treatment (i = SILc, LATc, LAT, ERL), $1_{\{Grass\}}$ is an indicator function [0 if treatment contains silage (SILc) and 1 if treatment contains fresh grass (LATc, LAT, ERL)], w_{ijk} is week number of the experiment for observation $k = 1, \dots, n_{ij}$ ($n_{ij} = 2$) of cow ($j = 1, \dots, 16$) with treatment i. Each cow tested two treatments and thus had four observations, and therefore the total number of observations was 64. Moreover, the random effect of cow ν_j and the residual error ε_{ijk} were assumed to be normal distributed with zero mean and variances σ_ν^2 and σ_ε^2, respectively.

Dataset 2 included the response variables for ATTD, chewing time, rumen SCFA proportions, milk FA and protein composition, and milk coagulation properties (Table 4, 6, and 7) measured with one observation within each experimental period (during digestibility measurement). Dataset 3 included the response variables for gas exchange (Table 5) measured also with one observation within each experimental period (during CH_4 measurement). For both dataset 2 and 3, the model in Equation 1 was also used, but since $n_{ij} = 1$, each cow had two observations, and therefore the total number of observations was 32 for each of datasets 2 and 3. Observations from two cows were, however, omitted due to occurrence of illness (treatment LATc and SILc in period 1 and LAT and LATc in period 2). *P*-values are given in the text as P_{Treat} (effect of treatment), $P_{\text{Grass} \times \text{w}}$ (linear effect of week number in experiment for treatments with fresh grass), $P_{\text{Grass} \times \text{wsqr}}$ (quadratic effect of week number in experiment for treatments with fresh grass relative to SILc), and P_{adj} (pairwise

comparisons between treatments with adjustment for multiple testing using Tukey's procedure). The linear and quadratic effects of week allows the difference relative to SILc to change linearly, concavely, or convexly over time for treatments with fresh grass. Least squares means with the highest corresponding standard error of mean (**SEM**) are reported in tables. Statistically significant difference was considered when P-value ≤ 0.05 and tendency when 0.05 < P-value ≤ 0.10.

RESULTS

Forages

Table 1 shows the chemical composition of the forages. The chemical analyses indicated that WSC concentration of fresh grass was 201-206 g/kg of DM whereas only 4 g/kg of DM for silage. The CP concentration was 122 g/kg of DM in grass harvested at late developmental stage compared to 152-158 g CP/kg of DM in silage and grass harvested at early developmental stage. Moreover, OMD determined in vitro for silage was 1.6 and 4.9%-unit higher compared to fresh grass harvested at early and late developmental stage, respectively. Estimates from NorFor showed negative PBV for grass harvested at late developmental stage. Limited differences in the FA composition between forages were observed (Table 2), whereas concentrates compared to forages generally had higher proportions of C18:1 *cis*-9 and C18:2 *cis*-6 and higher n6:n3 ratio but lower proportions of C16, C18:3 n3, and ∑PUFA.

Feed intake and overall milk production

Relative to SILc, cows fed treatments with fresh grass had a convex pattern for total DMI ($P_{Grass \times wsqr}$ = 0.03; Table 3; Figure 1A). No difference was observed for DMI for SILc compared to LATc, whereas DMI was higher (P_{adj} < 0.01 and P_{adj} = 0.02, respectively), when supplementing concentrates (LATc vs. LAT) and feeding ERL compared to LAT. The NDF intake was not different between treatments, and relative to SILc, cows fed treatments with fresh grass had a

constant NDF intake (Figure 1B), whereas iNDF intake increased linearly ($P_{Grass \times w} < 0.01$) for treatments with fresh grass relative to SILc (Figure 1C). Intake of CP was 17% lower ($P_{adj} = 0.03$) in LATc compared to SILc, and 39% higher ($P_{adj} < 0.01$) in ERL compared to LAT (Table 3).

Milk yield varied from 21.1 to 28.6 kg/d and was higher ($P_{adj} = 0.01$) in SILc compared to LATc, higher ($P_{adj} < 0.01$) in LATc compared to LAT, and higher ($P_{adj} = 0.01$) in ERL compared to LAT. The ECM yield varied from 21.5 to 27.3 kg/d, and was higher ($P_{adj} < 0.01$) for LATc compared to LAT. The milk protein yield was 174 g/d higher ($P_{adj} < 0.01$) and the milk fat concentration was 5.5 g/kg lower ($P_{adj} < 0.01$) in LATc compared to LAT. Overall, feed efficiency estimated as kg ECM/kg of DMI was not different between treatments, but compared to SILc, treatments with fresh grass showed a concave pattern ($P_{Grass \times wsqr} < 0.01$; Figure 1D). The NUE was not different between SILc and LATc, between LATc and LAT, and between LAT and ERL, but was negatively correlated to CP concentration of the diet (Pearson correlation coefficient = -0.85; $P < 0.01$; data not shown).

ATTD, Chewing Activity, and Rumen Fluid Composition

Feeding cows LATc resulted in a 3.4 and 6.0%-unit lower ($P_{adj} = 0.04$ and $P_{adj} < 0.01$, respectively) ATTD of OM compared to SILc and LAT, respectively, whereas no difference in ATTD of OM was detected between LAT and ERL (Table 4). Cows fed LAT spent 29 and 14% more ($P_{adj} < 0.01$ and $P_{adj} = 0.01$, respectively) time chewing while eating per kg of DMI compared to LATc and ERL, whereas no differences were shown between SILc and LATc for time spent chewing while eating and ruminating per kg of DMI. Except from isovalerate, the proportion of all SCFA in rumen fluid was affected by treatment. A higher ($P_{adj} = 0.02$) proportion of butyrate was detected in LATc compared to LAT, whereas no difference in proportions of butyrate were found in SILc and LATc. A higher ($P_{adj} < 0.01$) proportion of acetate in rumen fluid was detected in LATc compared to SILc, whereas the proportion of propionate in rumen fluid was higher ($P_{adj} = 0.02$) in

SILc compared to LATc resulting in a higher ($P_{adj} < 0.01$) acetate:propionate ratio for LATc compared to SILc. The proportion of each analyzed SCFA was not different between LAT and ERL.

Gas Exchange

There was no difference in neither CH_4 production (L/d), yield (L/kg of DMI), nor intensity (L/kg of ECM) between SILc and LATc, whereas CH_4 yield was 18% higher ($P_{adj} = 0.01$) for LAT compared to LATc (Table 5). Relative to SILc, cows fed fresh grass showed a convex pattern ($P_{Grass \times wsqr} = 0.01$) for CH_4 intensity (Figure 1E). Furthermore, CH_4 production, yield, and intensity were not different between ERL and LAT.

Milk Fatty Acid Composition

Compared to SILc, LATc had a lower ($P_{adj} < 0.01$, $P_{adj} = 0.02$, and $P_{adj} < 0.01$, respectively) FA proportion in milk of ∑C4-C10, ∑C12-C14, and ∑C16, and a higher ($P_{adj} < 0.01$, $P_{adj} < 0.01$, and $P_{adj} = 0.01$, respectively) FA proportion of ∑C18, ∑PUFA, and ∑n6 (Table 6). No overall treatment effect was observed on the proportion of ∑n3 in milk, whereas relative to SILc, treatments with fresh grass resulted in a linear decrease ($P_{Grass \times w} < 0.01$) in proportion of ∑n3 (Figure 1F). No overall significant differences in milk FA composition were observed among LATc, LAT, and ERL, except that C18:2 *cis*-6, ∑n6, and the n6:n3 ratio were higher ($P_{adj} = 0.03$, $P_{adj} = 0.03$, and $P_{adj} < 0.01$, respectively) in LATc compared to LAT.

Concentration of Vitamins in Milk, Milk Protein Composition, and Coagulation Properties

Treatment affected concentrations of urea ($P_{Treat} < 0.01$) and retinol ($P_{Treat} < 0.01$) in milk (Table 7). Compared to LATc, ERL had a higher ($P_{adj} = 0.01$) concentration of urea, whereas SILc and LAT resulted in intermediate concentrations. Compared to SILc, LATc had a higher

(P_{adj} = 0.02) concentration of retinol. Feeding treatments with fresh grass resulted in a convex pattern ($P_{Grass \times wsqr} < 0.01$) in concentration of α-tocopherol and riboflavin in milk (Figure 1G and 1H, respectively). For relative protein composition, treatment affected (P_{Treat} = 0.03 and P_{Treat} = 0.04, respectively) α_{S1}-CN 9P and phosphorylation degree of α_{S1}-CN (**PD**) and resulted in higher (P_{adj} = 0.03 for both) proportions of α_{S1}-CN 9P and PD in LAT compared to SILc, whereas LATc and ERL resulted in intermediate proportions. Treatment also affected (P_{Treat} = 0.02 and P_{Treat} = 0.05, respectively) α-LA and β-LG, where lower (P_{adj} = 0.02 and P_{adj} = 0.03, respectively) proportions of α-LA and β-LG were found for LAT compared to LATc. For coagulation properties, treatments did not affect curd firming rate (**CFR**) and maximal gel strength (**Gmax**), whereas rennet coagulation time (**RCT**) was affected (P_{Treat} = 0.02), showing shorter ($P_{adj} < 0.01$) RCT for SILc compared to ERL, with LATc and LAT having intermediate RCT.

DISCUSSION

The OMD for grass silage and the grass harvested at late developmental stage was intended to be similar, and grass harvested at early developmental stage should have a higher OMD. However, this was not attained, as the OMD determined in vitro for fresh grass harvested at both early and late developmental stage were 1.6 and 4.9 %-units, respectively, lower than silage. Changing weather conditions led to variation in grass development, but the difference between grass harvested at early and late developmental stage in e.g. OMD, stem proportion, and NDF concentration remained (as planned) constant throughout the experiment as reported in Hansen et al. (2020). Fertilization with N in the field, where fresh grass was harvested daily, was supplied following Danish standards. However, the CP concentration in harvested grass was lower than expected, resulting in slightly lower CP concentration for grass harvested at early developmental stage compared to silage, and even lower for grass harvested at late developmental stage. Based on expected nutrient composition of the grass harvested at late developmental stage, we designed the

445 rapeseed cake based concentrate pellet to fulfill requirements of energy and protein for cows fed
446 LATc and SILc, whereas treatments of ERL and LAT illustrated scenarios, where farmers would
447 feed fresh grass only.
448
449 ***Feed intake and ATTD***
450 Compared to the fresh grass harvested daily, less variation in DM and nutrient concentration of
451 the silage was expected, since the ensiled grass was from one field and one harvest time. Relative to
452 SILc, DMI of LATc, LAT, and ERL displayed variation throughout the experiment (Figure 1A).
453 The DMI of cows fed treatments with fresh grass reflected the changes in stem proportion, NDF
454 concentration, and the OMD of the grasses harvested throughout the experiment (Hansen et al.,
455 2020), i.e. DMI decreased towards the middle of the experiment and increased towards the end of
456 the experiment. Relative to SILc, cows fed treatments with fresh grass had similar NDF intake
457 throughout the experiment (Figure 1B) as the changes in NDF concentration of fresh grasses during
458 the experiment counteracted the changes in DMI. The constant NDF intake indicated that NDF
459 plays a key role in physical regulation of the voluntary DMI (Huhtanen et al., 2016). In contrast to
460 NDF intake, intake of iNDF increased during the experiment for cows fed treatments with fresh
461 grass compared to SILc (Figure 1C), which might be caused by enhanced lignification with rising
462 temperatures from spring to summer (Buxton, 1996) in combination with the observed increased
463 DMI in the last part of the experiment. The ATTD of OM for LATc was 3.4%-units lower
464 compared to SILc, which reflected the 4.9%-unit lower OMD determined in vitro for fresh grass
465 harvested at late developmental stage compared to silage.
466 Supplementation of concentrates in the current experiment (LATc vs. LAT) resulted in higher
467 CP concentration of the diet (137 vs. 121 g/kg of DM, respectively) but it also reduced ATTD of
468 OM by 6%-units (1%-unit for every kg/d increase in concentrates). For comparison, in a meta-
469 analysis using 142 diets from 59 studies, Nousiainen et al. (2009) showed only a 0.32%-unit

decrease in ATTD of OM for every kg/d increase in concentrates supplemented mainly separately to silages. However, Alstrup et al. (2016) reported that ATTD of OM was not different between rations with 20 or 50% concentrate (on DM basis) fed as a TMR with grass-clover silage. The ATTD of OM observed for LATc was therefore much lower than expected compared to the ATTD of OM for LAT. Rate of feed particle breakdown and rate of nutrient digestion can affect OM digestibility. The time spent chewing during rumination, equal to the time where feed particles are drastically reduced in size to enhance microbial adherence, was not different for LAT and LATc. However, the time spent chewing while eating was higher for LAT compared to LATc, probably due to the higher grass proportion in total ration in LAT. Moreover, rapid fermentation of easily degradable nutrients in LATc compared to LAT might have reduced pH and thereby inhibited the cellulolytic bacteria activity, or the cellulolytic bacteria might have had substrate preference for the added easily digestible carbohydrates. Rumen pH was not measured in rumen fluid sampled from the intact animals in the current experiment, due to the risk of saliva contamination during sampling (Larsen et al., 2020). However, changes in SCFA composition of rumen fluid, e.g. higher butyrate proportion, suggested that the rumen environment was affected by concentrate supplementation. Furthermore, supplementation of 3 kg/d of concentrate to fresh perennial ryegrass has shown to increase the fractional rate of passage of iNDF from the rumen to the omasal canal by 13% (Dineen et al., 2020), suggesting less time for ruminal digestion, when concentrates were supplemented. However, passage rates were not assessed in the current experiment.

The treatments ERL and LAT reflected a production system, where milk is produced without supplementation of concentrate, and farmers thus tolerate expected reduced milk yields by receiving higher premium prices for milk products. In such a system, optimizing the regrowth period of grass can improve field yields, feed intake, and milk production. Indeed, compared to LAT, ERL resulted in higher intake of DM, OM, and CP. Due to the low concentration of CP in LAT, estimations by NorFor showed a negative PBV (Table 1), which suggested a negative impact on rumen

digestibility. Therefore, it was unexpected that ATTD of OM did not differ between ERL and LAT, also considering that OMD determined in vitro was 3.3%-units lower for LAT compared to ERL. Several factors might have contributed to reducing the difference of ATTD of OM between ERL and LAT. When using NorFor to recalculate the protein values of LAT based on the obtained feed intakes, estimation of the PBV was less negative for LAT (data not shown) compared to the estimation using the grass samples only (Table 1). This suggested that the expected negative effect of limited N supply on OM digestion in the rumen, calculated from the analyses of grass harvested at late development stage, was actually less severe if corrected for the observed feed intake. Moreover, rumen fluid SCFA proportions of ERL and LAT were not different and showed therefore no sign of difference in rumen fermentation patterns between the two treatments. The ATTD of OM of ERL and LAT might also not have been different due to the relatively higher DMI for ERL (Robinson et al., 1987). High intakes can be facilitated by high fractional rates of passage of OM out of the rumen, implying that potentially degradable OM could be lost before being digested in the rumen. Furthermore, and possibly with limited effect, cows fed LAT spent 14% more time chewing while eating compared to cows fed ERL, suggesting more efficient mastication of feed particles. This is in alignment with De Boever et al. (1993), who also observed that cows spent more time chewing while eating and ruminating when feeding silage harvested at late compared to early developmental stage. This was probably caused by the increased lignification and could potentially increase surface area for microbial adherence and thereby facilitate a more rapid increase in rate of digestion (Kennedy and Doyle, 1993). Improved mastication during eating for LAT in this experiment could probably not solely explain why ERL and LAT did not differ in ATTD of OM, but it might have contributed to diminishing the difference in combination with the obtained differences in DMI.

Gas exchange

Cows fed SILc and LATc did not differ in CH_4 production (L/d), yield (L/kg of DMI), and intensity (L/kg of ECM). However, we had expected higher CH_4 yield from cows fed LATc compared to SILc due to nutritional differences between the silage and fresh grass, and due to the observed higher acetate:propionate ratio in rumen fluid. Compared to silage, sugar in fresh grass has not been fermented during ensiling, indicated by the low concentration of WSC in silage compared to both types of fresh grass (Table 1). Sugar contributes to the production of butyrate in rumen liquid and the parallel formation of H_2, which is a precursor for the formation of CH_4 (Janssen, 2010). However, no difference was found in proportion of butyrate in rumen fluid between SILc and LATc, and despite large difference, H_2 production was not significantly higher for LATc. In contrast to our study, Younge et al. (2004) found a higher proportion of butyrate in rumen fluid, when feeding fresh perennial ryegrass compared to silage, presumably caused by the higher concentration of WSC in the fresh grass. In agreement with our study, Johansen et al. (2017) showed that increasing DM concentration of silage through prolonged wilting increased the concentration of total sugar in the silage, but without affecting the CH_4 yield. The CH_4 intensity for cows fed diets with fresh grass increased at the end of the experiment compared to cows fed SILc (Figure 1E). The increase was probably driven by a decrease in ECM yield and a concomitant increase in DMI, which would promote higher production of CH_4.

Supplementation of concentrate to fresh grass (LATc vs. LAT) had no effect on CH_4 production and intensity, whereas LATc compared to LAT had a lower CH_4 yield due to the higher DMI. Extending the regrowth period from three to five weeks had no effect on CH_4 production in the current experiment. Although not statistically different, CH_4 yield was 9% higher for LAT compared to ERL, which was higher than the 1% difference reported for silages by Warner et al. (2015) and lower than the 12% difference reported by Brask et al. (2013), where three and five weeks of regrowth were applied as early and late harvested forage also, respectively.

Overall Milk Performance

The milk yield was unexpectedly 2.6 kg/d lower for LATc than SILc. This was probably caused by lower CP intake, lower ATTD of OM, and, although not statistically different, the 0.9 kg/d lower DMI for LATc. In contrast, Younge et al. (2004) showed at DMI comparable to our study that cows fed fresh grass compared to silage did not differ in DMI and despite not being significant, produced 1.5 kg of milk more per day. The supply of MP can increase milk yield, and Younge et al. (2004) showed that the omasal flows of non-ammonia N and of non-ammonia non-microbial N were higher, when feeding fresh grass compared to silage. For comparison, estimation of AAT performed in NorFor was higher in fresh grass compared to silage in our experiment (Table 1). However, lower ATTD of OM in LATc compared to SILc might have caused MP supply from LATc to be smaller than expected and thus be a possible explanation for the reduced milk yield in our study. Compared to SILc, LATc had higher concentration of WSC, which is a key nutrient serving as a substrate for the microbial protein synthesis in the rumen. Miller et al. (2001) compared two rations of fresh grass differing in concentration of WSC and found, in contrast to our study, that milk yield was 2.7 kg/d higher, when feeding the diet high in WSC. However, compared to the silage and fresh grass used in our study, both treatments were of fresh grass.

Relative to milk yield, the difference in ECM yield was smaller when concentrate was supplemented to fresh grass (LATc vs. LAT), and the higher milk fat concentration for LAT had most likely caused the diminishing effects, when milk yield was corrected for energy. Generally, concentrations of protein and fat increase and decrease, respectively, when the proportion of concentrates in the ration is increased (Bargo et al., 2003). For milk protein, we only observed higher yield and not concentration, when supplementing concentrates. As reviewed by Bargo et al. (2003), the relatively high response in milk yield from supplementation of concentrates (LATc vs. LAT) was in agreement with the relatively low substitution rate of 0.52 kg/kg obtained in our experiment, since those parameters are negatively correlated.

570 Compared to LAT, milk yield was 2.3 kg higher for ERL, and despite not being significant,
571 ECM yield was 1.9 kg/d higher. The numerically higher concentration of fat in LAT might have
572 contributed to diminishing the difference in yield if expressed as ECM. Despite the large variation
573 in means of NUE, there was no significant difference between SILc and LATc, between LATc and
574 LAT, or between LAT and ERL. However, the Pearson correlation coefficient of the linear
575 relationship across all observations between NUE and the concentration of CP in the treatments was
576 negative, which is in agreement with Yan et al. (2006), and could possibly explain, why NUE was
577 higher for LAT compared to SILc.
578
579 ***Milk Fatty Acids***
580 Higher proportions of ∑C4-C10, ∑C12-C14, and ∑C16 in milk FA suggested higher de novo
581 synthesis in cows fed SILc compared to LATc and, moreover, we observed lower proportions of
582 ∑C18 and especially C18:1 *cis*-9 and C18:2 *cis*-6 in SILc compared to LATc. For comparison, De
583 La Torre-Santos et al. (2020) reported no differences in proportions of C18:1 and C18:2 in milk
584 from cows fed fresh grass compared to silage. Relative to SILc, the proportion of ∑n3 FA in milk
585 from cows fed treatments with fresh grass was higher in the beginning of the experiment and
586 decreased to a similar level in the end of the experiment, mainly driven by a reduction in the
587 proportion of C18:3 n3. Overall, the proportion of ∑n3 FA in milk from cows fed treatments with
588 fresh grass (0.90 to 1.01 g/100 g FA) was comparable to previous studies, e.g. 0.88 g/100 g FA
589 (Stergiadis et al., 2014), 0.6 g/100 g FA (O'Callaghan et al., 2018), and 1.57 g/100g FA (Benbrook
590 et al., 2018). In human nutrition, the n6:n3 ratio has been suggested to be 15 times higher compared
591 to the nutritional optimum of about 1 (Benbrook et al., 2018), and the current study showed that the
592 n6:n3 ratio in milk could be reduced if cows were fed grass only (LAT vs. LATc) or grass silage
593 instead of fresh grass (SILc vs. LATc). The beneficial effect was obtained mainly through smaller
594 proportions of ∑n6 in milk from LAT compared to LATc and in milk from SILc compared to

595 LATc. The higher proportion of C18:2 *cis*-6 was the main driver for higher proportion of ∑PUFA
596 in LATc compared to SILc. Feeding fresh forage rather than concentrate based TMR has been
597 suggested to increase the activity of stearoyl CoA desaturase (O'Callaghan et al., 2018). However,
598 the proportion of CLA *cis*-9, *trans*-11 was not observed to differ between SILc and LATc.
599
600 ***Minor Milk Constituents, Milk Protein Composition, and Coagulation Properties***
601 The change in concentration of α-tocopherol and riboflavin in milk throughout the experiment
602 reflected the parallel change in DMI, and mirrored the change in stem proportion of fresh grass
603 throughout the experiment. Concentrations of α-tocopherol and riboflavin are generally higher in
604 leaves compared to stems of grass (Booth, 1964; Ballet et al., 2000), suggesting that the high stem
605 proportion in the middle of the experiment (Hansen et al., 2020) caused the concentration of α-
606 tocopherol and riboflavin in milk to drop and subsequently increase again (Figure 1G and H). As
607 reviewed by Nozière et al. (2006), the concentration of retinol in milk is generally lower, when diets
608 consist of preserved forages, which was in alignment with the observed lower concentration of
609 retinol in SILc compared to all treatments with fresh grass.
610 An earlier study showed that higher CP concentration in the diet resulted in higher protein and
611 mineral concentrations, but lower relative concentration of α_{S1}-CN 9P (and thus PD) in milk
612 (Poulsen et al., 2021). In the present study, milk protein concentration was not affected by
613 treatment, but interestingly α_{S1}-CN 9P (and PD) was affected, which again suggests that not only
614 genetic variation but also feeding may affect this trait in milk. The documented variation in α-LA
615 and β-LG relative to treatment suggest that concentrate supplementation increases the relative
616 proportion of both traits. This in turn affects casein number, which is an important parameter
617 relative to cheese making, where increasing casein number is associated with cheese yield
618 (Wedholm et al., 2006).

What drives variation in RCT between treatments is less clear, but impaired milk coagulation properties have previously been related to subtle differences in PD (Jensen et al., 2012), where longer RCT tended to be associated with higher PD. This somewhat confirms that poor milk coagulation properties may be related to higher relative concentration of α_{S1}-CN 9P and thus higher PD, as observed in milk from LAT and ERL. However, factors affecting milk coagulation properties may differ between RCT, defining the clot time of the milk coagulation compared to CFR and Gmax, which describe the development of the protein gel network and the increasing strength after initial coagulation.

CONCLUSIONS

This study showed that managing grass feeding of dairy cows affected DMI, milk yield, milk quality, and gas exchange. Replacing silage with fresh grass resulted in no difference in DMI, ECM yield, and CH_4 yield, although OM digestibility was lower for the treatment with fresh grass. Harvest of fresh grass at late compared to early developmental stage decreased ECM yield. Cows fed grass harvested at early developmental stage could not achieve ECM yields similar to cows fed fresh grass harvested at late developmental stage supplemented with 6 kg of concentrates. Supplementation of concentrates decreased CH_4 yield but not CH_4 intensity, and the developmental stage at harvest had no effect on CH_4 yield or intensity. Supplementation of concentrates or replacing fresh grass with silage reduced the n6:n3 ratio of FA in milk, and replacement of fresh grass with silage increased the de novo synthesis of FA.

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Table 1. Ingredient composition (g/kg) for concentrate and marker pellets, and chemical composition (mean ± SD; g/kg of DM unless otherwise stated) of forages (n = 10), concentrate (n = 2) and marker pellets (n = 2).

	Silage[1]	Early	Late	Concentrate pellet	Marker Pellet
Ingredients					
Wheat				691	435
Rape seed cake				236	149
Sugar beet molasses				73	83
Titanium dioxide					43
Vitamin[2]					33
Mineral[3]					257
Nutrient composition					
DM, g/kg	273 ± 5.4	200 ± 37.3	187 ± 18.5	885 ± 6.6	926 ± 1.5
Ash	90.2 ± 3.40	75.6 ± 5.43	72.6 ± 3.39	33.5 ± 0.71	341 ± 2.7
CP	158 ± 7.7	152 ± 41.4	122 ± 21.0	173 ± 0.2	119 ± 2.2
Soluble N, g/kg of N	629 ± 19.1	308 ± 33.8	332 ± 37.1	331 ± 3.4	287 ± 7.2
Glucose	0.933 ± 0.3322	45.0 ± 15.97	56.8 ± 12.94	NA[4]	NA
Fructose	1.68 ± 1.073	48.6 ± 7.03	51.3 ± 6.39	NA	NA
Sucrose	0.817 ±0.2326	24.0 ± 32.57	23.8 ± 24.96	NA	NA
Fructan	0.362 ± 0.8994	83.3 ± 59.15	74.2 ± 44.56	NA	NA
WSC[5]	3.79 ± 1.341	201 ± 73.5	206 ± 59.2	NA	NA
NDF	403 ± 5.0	447 ± 35.0	491 ± 45.1	152 ± 5.4	112 ± 5.2
ADF	253 ± 4.3	244 ± 22.1	277 ± 29.6	66.4 ± 2.97	57.8 ± 0.92
ADL	17.1 ± 2.90	17.6 ± 9.02	18.4 ± 6.37	20.9 ± 1.48	8.40 ± 0.849
iNDF[6]	43.1 ± 2.65	35.6 ± 5.67	53.7 ± 13.49	45.1 ± 0.07	26.9 ± 0.66
FA	17.4 ± 2.25	16.9 ± 3.89	14.6 ± 2.19	26.0 ± 1.72	19.7 ± 0.65
Titanium dioxide	ND[7]	ND	ND	0.330 ± 0.0861	43.4 ± 0.60
OMD,[8] %	79.3 ± 0.52	77.7 ± 3.07	74.4 ± 3.65	87.2 ± 0.92	86.7 ± 1.23
NEL,[9] MJ/kg of DM	6.45	6.53	6.19	7.68	5.20
AAT,[10] g/MJ of NEL	80	95	90	114	79
PBV,[11] g/kg of DM	27	4	-16	2	3

[1]Mean fermentation characteristics for silage (n = 10): pH = 3.89; L-lactate = 74.1 g/kg of DM; acetate = 46.9 g/kg of DM; propionate = 6.3 g/kg of DM; butyrate = 2.8 g/kg of DM; ammonia N = 75 g/kg of total N.

[2]Supplex ADE (Vilofoss).

[3]VM2 grøn (Vilofoss).

[4]Not analyzed.

[5]Water soluble carbohydrates (sum of glucose, fructose, sucrose, and fructan).

[6]Indigetible NDF.

[7]Not detected.

[8]In vivo OM digestibility calculated as 4.10 + 0.959 × in vitro OM digestibility for forages and calculated as 5.38 + 0.867 × enzymatic OM digestibility for pellets.

[90]NEL_{20}, net energy for lactation, calculated in NorFor according to Volden (2011).

[10]AA absorbed in the small intestine, calculated in NorFor according to Volden (2011).

[11]Protein balance in the rumen, calculated in NorFor according to Volden (2011).

Table 2. Fatty acid (FA) composition (mean ± SD; g/100 g FA) of forages (n = 10), concentrate (n = 2) and marker pellets (n = 2).

Item[1]	Silage	Early	Late	Concentrate pellet	Marker Pellet
C16	16.0 ± 0.26	16.5 ± 1.01	18.1 ± 1.31	9.58 ± 0.151	11.5 ± 0.12
C18	1.74 ± 0.094	1.64 ± 0.223	1.65 ± 0.179	1.38 ± 0.007	1.67 ± 0.027
C18:1 *cis*-9	2.44 ± 1.094	2.16 ± 0.412	2.45 ± 0.533	39.9 ± 0.75	39.3 ± 0.02
C18:2 *cis*-6	14.5 ± 0.27	13.6 ± 0.94	15.0 ± 1.53	35.0 ± 0.78	34.7 ± 0.03
C18:3 n3	59.3 ± 1.43	60.6 ± 2.33	56.6 ± 3.39	7.42 ± 0.057	6.23 ± 0.091
∑C4-C14	1.47 ± 0.164	0.937 ± 0.3053	1.02 ± 0.244	0.125 ± 0.0095	0.220 ± 0.0025
∑C16	17.1 ± 0.23	17.5 ± 0.96	19.2 ± 1.26	10.1 ± 0.15	11.9 ± 0.11
∑C18	78.5 ± 0.37	78.4 ± 1.37	76.2 ± 1.62	87.9 ± 0.06	85.9 ± 0.11
∑PUFA	74.1 ± 1.21	74.6 ± 1.56	72.1 ± 2.05	42.5 ± 0.72	41.1 ± 0.06
∑n3	59.4 ± 1.43	60.8 ± 2.30	56.9 ± 3.30	7.42 ± 0.057	6.23 ± 0.091
∑n6	14.6 ± 0.27	13.7 ± 0.95	15.1 ± 1.37	35.1 ± 0.77	34.9 ± 0.03
n6:n3 ratio	0.247 ± 0.0104	0.225 ± 0.0236	0.267 ± 0.0402	4.73 ± 0.140	5.60 ± 0.087

[1]Other individual FA not mentioned but included in the total sum: C4, C6, C8, C10, C11, C12, C13, C14, C14:1, C15, C16:1, C17, C17:1, phytanic acid, C18:1 trans, C18:1 trans-9, C18:1 trans-11, C18:2 trans-6, CLA *cis*-9 *trans*-11, C18:3 n6, C20, C20:1, C20:2, C20:3 n3, C20:3 n6, C20:4 n6, C20:5 n3, C21, C22, C22:1 n9, C22:2, C22:6 n3, C23, C24, and C24:1.

Table 3. Intake and overall milk production in dairy cows fed one of four treatments differing in forage type and concentrate supplementation.

	Treatment[1]				SEM[2]	P-values[3]		
	SILc	LATc	LAT	ERL		Treat	Grass × w	Grass × wsqr
n_{cow}	7	6	7	8				
$n_{samples}$	14	12	14	16				
Intake, kg/d								
Forage DM	12.2^{bc}	11.4^{c}	14.1^{ab}	15.0^{a}	0.60	<0.01	0.82	0.05
Pellet DM[4]	5.87	5.87	0.556	0.556				
Total DM	17.8^{a}	16.9^{a}	14.1^{c}	15.5^{b}	0.45	<0.01	0.06	0.03
OM	16.3^{a}	15.7^{a}	12.9^{c}	14.2^{b}	0.41	<0.01	0.06	0.02
CP	2.84^{a}	2.35^{b}	1.68^{c}	2.33^{b}	0.126	<0.01	0.24	0.84
FA	0.334^{a}	0.298^{ab}	0.200^{c}	0.257^{b}	0.0129	<0.01	0.07	0.48
NDF	5.62	6.26	6.46	6.76	0.256	0.06	0.43	0.35
ADF	3.37	3.43	3.62	3.67	0.153	0.60	0.17	0.43
ADL	0.309	0.320	0.266	0.296	0.0298	0.62	0.04	0.69
iNDF[5]	0.756^{ab}	0.835^{a}	0.691^{b}	0.538^{c}	0.0365	<0.01	<0.01	0.77
Milk yield								
Milk, kg/d	28.6^{a}	26.0^{b}	21.1^{d}	23.4^{c}	1.16	<0.01	0.02	0.81
ECM, kg/d	27.3^{a}	25.3^{ab}	21.5^{c}	23.4^{bc}	1.03	<0.01	<0.01	0.48
Lactose, g/d	1372^{a}	1256^{b}	992^{d}	1099^{c}	57.4	<0.01	<0.01	0.82
Protein, g/d	897^{a}	836^{a}	661^{c}	748^{b}	28.7	<0.01	0.02	0.37
Fat, g/d	1080	1007	913	968	47.1	0.06	<0.01	0.15
Milk composition								
Lactose, g/kg	48.0^{a}	48.0^{a}	47.2^{ab}	46.9^{b}	0.33	0.01	<0.01	0.53
Protein, g/kg	31.5	32.3	31.7	32.6	0.79	0.07	0.95	0.15
Fat, g/kg	38.1^{b}	38.2^{b}	43.7^{a}	42.0^{a}	1.20	<0.01	<0.01	0.03
kg of ECM/kg of DMI	1.54	1.50	1.55	1.52	0.055	0.46	<0.01	<0.01
NUE,[6] %	30.6^{b}	37.0^{ab}	38.8^{a}	32.6^{ab}	1.95	<0.01	0.83	0.91

$^{a\text{-}c}$Values within the same line with different superscripts differ ($P \leq 0.05$).

[1]SILc = Silage + 6 kg concentrate pellets; LATc = Fresh grass harvested after 5 weeks regrowth + 6 kg concentrate pellets; LAT = Fresh grass harvested after 5 weeks regrowth; ERL = Fresh grass harvested after 3 weeks regrowth; all treatments were supplemented 0.6 kg/d of marker pellets.

[2]Highest SEM is given.

[3]Treat = effect of treatment; Grass × w = linear effect of week number in experiment for treatments with fresh grass relative to SILc; Grass × wsqr = quadratic effect of week number in experiment for treatments with fresh grass relative to SILc.

[4]No statistics provided since a fixed amount of concentrate and marker pellets were given separately twice daily and no refusals we registered.

[5]Indigestible NDF.

[6]Nitrogen use efficiency calculated as N milk / N intake × 100%.

Table 4. Digestibility, chewing time, and proportion of short chain fatty acids (SCFA) in rumen fluid of dairy cows fed one of four treatments differing in forage type and concentrate supplementation.

	Treatment[1]				SEM[2]	P-values[3]		
	SILc	LATc	LAT	ERL		Treat	Grass × w	Grass × wsqr
n	7	6	7	8				
Apparent total tract digestibility, %								
DM	75.9[a]	72.6[b]	78.9[a]	77.9[a]	0.96	<0.01	0.66	0.84
OM	77.4[a]	74.0[b]	80.0[a]	79.4[a]	0.95	<0.01	0.80	0.56
Chewing time, min/day								
Eating	541	548	608	556	24.5	0.15	0.62	0.54
Rumination	515	517	493	495	14.6	0.30	<0.01	0.23
Chewing time, min/kg DMI								
Eating	29.2[c]	32.2[c]	41.6[a]	36.4[b]	1.32	<0.01	0.59	<0.01
Rumination	28.6[b]	30.1[ab]	34.0[a]	31.9[ab]	1.33	0.03	0.06	0.26
SCFA proportions, mol/100 mol of total SCFA								
L-lactate	0.570	0.098	0.0415	0.101	0.2359	0.54	0.80	0.95
Acetate	58.7[b]	64.4[a]	65.7[a]	66.0[a]	0.84	<0.01	0.60	0.06
Propionate	20.6[a]	18.2[b]	19.3[ab]	18.9[ab]	0.57	0.03	0.08	0.52
Isobutyrate	0.925[a]	0.732[b]	0.839[ab]	0.944[a]	0.0453	<0.01	0.76	<0.01
Butyrate	14.9[a]	13.5[a]	11.7[b]	11.2[b]	0.44	<0.01	0.71	<0.01
Isovalerate	1.67	1.07	1.08	1.11	0.187	0.82	0.70	0.04
Valerate	1.96[a]	1.46[b]	1.13[c]	1.25[c]	0.056	<0.01	0.77	0.36
Caproate	0.703[a]	0.552[a]	0.329[b]	0.281[b]	0.0484	<0.01	<0.01	0.19
Acetate:propionate ratio	2.86[b]	3.59[a]	3.43[a]	3.50[a]	0.138	<0.01	0.14	0.22

[a-c]Values within the same line with different superscripts differ ($P \leq 0.05$).

[1]SILc = Silage + 6 kg concentrate pellets; LATc = Fresh grass harvested after 5 weeks regrowth + 6 kg concentrate pellets; LAT = Fresh grass harvested after 5 weeks regrowth; ERL = Fresh grass harvested after 3 weeks regrowth; all treatments were supplemented 0.6 kg/d of marker pellets.

[2]Highest SEM is given.

[3]Treat = effect of treatment; Grass × w = linear effect of week number in experiment for treatments with fresh grass relative to SILc; Grass × wsqr = quadratic effect of week number in experiment for treatments with fresh grass relative to SILc.

Table 5. Gas exchange in dairy cows fed one of four treatments differing in forage type and concentrate supplementation.

	Treatment[1]				SEM[2]	P-values[3]		
	SILc	LATc	LAT	ERL		Treat	Grass × w	Grass × wsqr
n	7	6	7	8				
Gas production, L/d								
CH_4	490[a]	454[ab]	423[b]	453[ab]	16.9	0.02	0.30	0.13
CO_2	6346[a]	6208[a]	5312[b]	6051[a]	114.3	<0.01	0.19	0.19
O_2	5815[a]	5590[a]	4979[b]	5573[a]	107.8	<0.01	0.97	0.59
H_2	7.43	17.0	10.7	10.2	2.307	0.10	0.30	0.04
RQ[4]	1.10[a]	1.11[a]	1.07[b]	1.08[ab]	0.011	0.01	<0.01	<0.01
CH_4:CO_2 ratio	0.0773[a]	0.0727[a]	0.0794[a]	0.0748[a]	0.00192	0.04	0.37	0.29
CH_4 yield[5]	28.5[b]	27.2[b]	31.6[a]	29.2[ab]	0.85	0.01	0.08	0.34
CH_4 intensity[6]	18.4	18.3	20.8	20.2	0.77	0.09	<0.01	0.01

[a-b]Values within the same line with different superscripts differ ($P \leq 0.05$).

[1]SILc = Silage + 6 kg concentrate pellets; LATc = Fresh grass harvested after 5 weeks regrowth + 6 kg concentrate pellets; LAT = Fresh grass harvested after 5 weeks regrowth; ERL = Fresh grass harvested after 3 weeks regrowth; all treatments were supplemented 0.6 kg/d of marker pellets.

[2]Highest SEM is given.

[3]Treat = effect of treatment; Grass × w = linear effect of week number in experiment for treatments with fresh grass relative to SILc; Grass × wsqr = quadratic effect of week number in experiment for treatments with fresh grass relative to SILc.

[4]Respiration quotient.

[5]L CH_4/kg of DM intake.

[6]L CH_4/kg of energy corrected milk.

Table 6. Fatty acid (FA) proportion (g/100 g total milk FA unless otherwise stated) of milk from dairy cows fed one of four treatments differing in forage type and concentrate supplementation.

	Treatment[1]				SEM[2]	*P*-values[3]		
	SILc	LATc	LAT	ERL		Treat	Grass × w	Grass × wsqr
n	7	6	7	8				
C4	6.82	6.52	6.44	6.61	0.165	0.03	0.65	0.01
C6	3.21^{a}	2.76^{b}	2.57^{b}	2.62^{b}	0.084	<0.01	0.48	<0.01
C8	1.65^{a}	1.31^{b}	1.12^{b}	1.17^{b}	0.059	<0.01	0.61	<0.01
C10	3.76^{a}	2.79^{b}	2.21^{b}	2.42^{b}	0.168	<0.01	0.90	<0.01
C11	0.0943^{a}	0.0330^{b}	0.0152^{b}	0.0225^{b}	0.00892	<0.01	0.99	0.01
C12	4.24^{a}	3.04^{b}	2.41^{b}	2.60^{b}	0.21	<0.01	0.99	<0.01
C13	0.144^{a}	0.0881^{b}	0.0797^{b}	0.0712^{b}	0.00788	<0.01	0.33	0.03
C14	12.6^{a}	10.7^{b}	9.40^{b}	9.94^{b}	0.477	<0.01	0.49	0.01
C14:1	1.21	1.09	1.04	0.934	0.1018	0.09	0.02	0.16
C15	1.29^{a}	0.971^{b}	1.07^{b}	0.970^{b}	0.0597	<0.01	<0.01	0.07
C16	29.0^{a}	25.3^{b}	26.7^{b}	25.7^{b}	0.72	<0.01	0.03	0.18
C16:1	1.66^{b}	2.24^{ab}	2.63^{a}	2.33^{a}	0.20	<0.01	0.47	0.01
C17	0.512^{b}	0.576^{ab}	0.649^{a}	0.667^{a}	0.0274	0.01	0.21	0.88
C17:1	0.219^{c}	0.328^{b}	0.448^{a}	0.392^{ab}	0.0241	<0.01	0.24	<0.01
C18	8.87	10.1	10.1	11.1	0.542	0.06	0.12	0.74
C18:1 *cis*-9	18.7^{b}	24.7^{a}	27.3^{a}	25.1^{a}	1.33	<0.01	0.74	<0.01
C18:2 *cis*-6	1.37^{b}	1.78^{a}	1.44^{b}	1.39^{b}	0.086	<0.01	0.01	0.04
CLA *cis*-9, *trans*-11	0.653	0.854	0.749	0.905	0.1105	0.63	0.01	0.33
C18:3 n3	0.776^{b}	0.813^{ab}	0.883^{ab}	0.931^{a}	0.0495	0.05	<0.01	0.60
Other FA[4]	3.16	3.88	3.20	3.90	0.342	0.33	0.11	0.30
∑C4-C10	15.4^{a}	13.4^{b}	12.4^{b}	12.9^{b}	0.35	<0.01	0.60	<0.01
∑C12-C14:1	18.1^{a}	15.0^{b}	12.9^{b}	13.5^{b}	0.71	<0.01	0.43	<0.01
∑C16	30.5^{a}	27.5^{b}	29.5^{ab}	28.0^{b}	0.69	<0.01	0.01	0.67
∑C18	31.8^{b}	40.1^{a}	42.1^{a}	41.6^{a}	1.53	<0.01	0.90	0.01
∑PUFA	3.18^{b}	3.79^{a}	3.46^{ab}	3.60^{a}	0.127	0.01	0.03	0.76
∑n3	0.874	0.902	0.953	1.01	0.0524	0.18	<0.01	0.99
∑n6	1.53^{b}	1.94^{a}	1.59^{b}	1.52^{b}	0.083	<0.01	<0.01	0.07
n6:n3 ratio	1.80^{b}	2.18^{a}	1.67^{b}	1.54^{b}	0.087	<0.01	0.11	0.05

$^{a-c}$Values within the same line with different superscripts differ ($P \leq 0.05$).

[1]SILc = Silage + 6 kg concentrate pellets; LATc = Fresh grass harvested after 5 weeks regrowth + 6 kg concentrate pellets; LAT = Fresh grass harvested after 5 weeks regrowth; ERL = Fresh grass harvested after 3 weeks regrowth; all treatments were supplemented 0.6 kg/d of marker pellets.

[2]Highest SEM is given.

[3]Treat = effect of treatment; Grass × w = linear effect of week number in experiment for treatments with fresh grass relative to SILc; Grass × wsqr = quadratic effect of week number in experiment for treatments with fresh grass relative to SILc.

[4]Other individual FA not mentioned but included in the total sum: phytanic acid, C18:1 trans, C18:1 trans-9, C18:1 trans-11, C18:2 trans-6, C18:3 n6, C20, C20:1, C20:2, C20:3 n3, C20:3 n6, C20:4 n6, C20:5 n3, C21, C22, C22:1 n9, C22:2, C22:6 n3, C23, C24, and C24:1.

Table 7. Concentration of minor metabolites, protein composition, and coagulation properties of milk from dairy cows fed one of four treatments differing in forage type and concentrate supplementation.

	Treatment[1]				SEM[2]	*P*-values[3]		
	SILc	LATc	LAT	ERL		Treat	Grass × w	Grass × wsqr
n	7	6	7	8				
Milk and protein composition								
MUN, mg/100 ml	9.01^{ab}	6.34^{b}	8.55^{ab}	12.5^{a}	1.406	<0.01	0.07	<0.01
Citrate, g/100 g	0.179	0.174	0.207	0.189	0.0107	0.17	0.12	0.72
pH	6.71	6.74	6.73	6.74	0.016	0.50	0.04	0.10
Conductivity, mS/cm	4.61	4.55	4.58	4.55	0.078	0.53	0.05	0.01
Minerals and vitamins								
Total Ca, mg/L	1410	1378	1504	1481	56.4	0.33	0.25	0.97
α-tocopherol, μg/mL	1.03	1.13	1.23	1.21	0.071	0.43	0.34	<0.01
Retinol, μg/g	0.622^{b}	0.779^{a}	0.800^{a}	0.799^{a}	0.0404	<0.01	0.98	0.06
Riboflavin, mg/L	1.41	1.34	1.46	1.37	0.110	0.08	0.01	<0.01
Protein composition,[4] % wt/wt of total protein in milk								
α_{S1}-CN	33.4	32.8	33.7	33.5	0.66	0.64	0.26	0.93
α_{S1}-CN 8P	24.7	24.3	23.9	24.3	0.62	0.59	0.87	0.52
α_{S1}-CN 9P	8.61^{b}	8.63^{ab}	9.72^{a}	9.29^{ab}	0.414	0.03	0.02	0.30
α_{S1}-CN PD	25.7^{b}	26.2^{ab}	28.9^{a}	27.7^{ab}	1.15	0.04	0.06	0.27
α_{S2}-CN	7.01	6.84	6.62	6.54	0.459	0.30	0.59	0.60
β-CN	38.8	39.4	39.9	40.1	0.73	0.51	0.32	0.40
κ-CN	8.81	8.80	8.78	8.47	0.648	0.80	0.44	0.32
G κ-CN	3.73	3.75	3.53	3.59	0.342	0.65	0.52	0.55
UG κ-CN	5.07	5.07	5.24	4.88	0.434	0.47	0.51	0.09
κ-CN GD	42.5	42.6	39.7	42.6	2.44	0.15	0.99	0.03
α-LA	3.26^{ab}	3.46^{a}	2.86^{b}	2.93^{b}	0.135	0.02	0.19	0.64
β-LG	8.51^{ab}	9.07^{a}	7.92^{b}	8.62^{ab}	0.467	0.05	0.44	0.76
Coagulation properties[5]								
RCT, min	16.2^{b}	16.9^{ab}	17.3^{ab}	18.4^{a}	1.15	0.02	0.32	0.07
CFR, Pa/min	14.8	11.2	12.5	7.64	2.302	0.11	0.13	0.12
Gmax, Pa	293	223	207	157	44.0	0.24	0.32	0.23

[a-b]Values within the same line with different superscripts differ ($P \leq 0.05$).

[1]SILc = Silage + 6 kg concentrate pellets; LATc = Fresh grass harvested after 5 weeks regrowth + 6 kg concentrate pellets; LAT = Fresh grass harvested after 5 weeks regrowth; ERL = Fresh grass harvested after 3 weeks regrowth; all treatments were supplemented 0.6 kg/d of marker pellets.

[2]Highest SEM is given.

[3]Treat = effect of treatment; Grass × w = linear effect of week number in experiment for treatments with fresh grass relative to SILc; Grass × wsqr = quadratic effect of week number in experiment for treatments with fresh grass relative to SILc.

[4]PD = Phosphorylation degree calculated as α_{S1}-CN 9P / total α_{S1}-CN × 100%; G κ-CN = Glycosylated κ-CN; UG κ-CN = Unglycosylated κ-CN; GD = Glycosylation degree calculated as G κ-CN / total κ-CN × 100%.

[5]RCT = Rennet coagulation time; CFR = Curd firming rate; Gmax = Maximum gel strength.

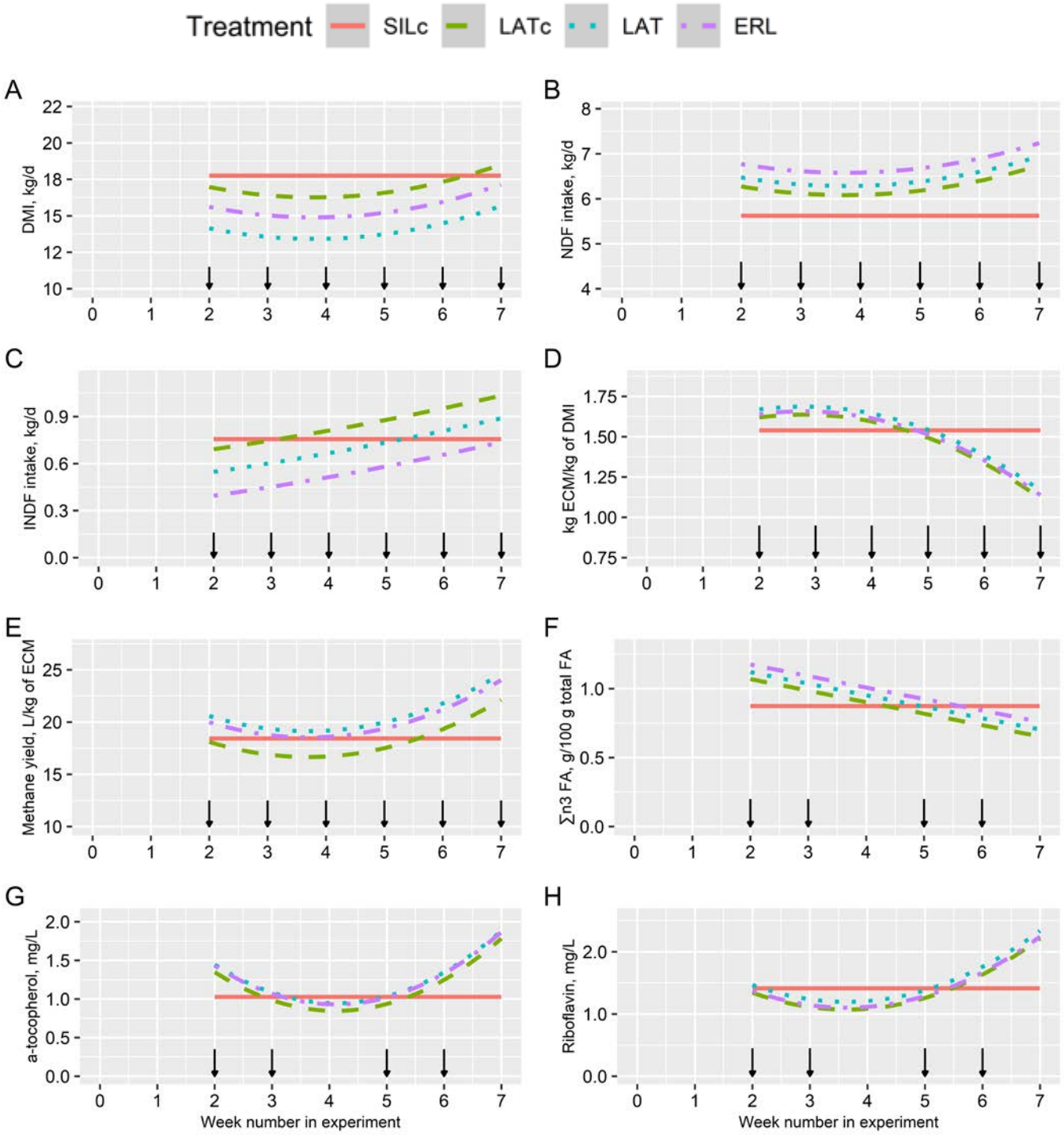

Figure 1: Interaction plots of A) DM intake (DMI), B) NDF intake, C) indigestible NDF intake, D) feed efficiency (kg ECM/kg of DMI), E) methane intensity, F) proportion of ∑n3 fatty acids (FA) in milk FA, G) milk concentration of α-tocopherol, and H) milk concentration of riboflavin in dairy cows fed one of four treatments differing in forage type and concentrate supplementation starting from first sampling until the end of the experiment (week 2 to 7; arrows indicate in which week number of the experiment the response variable in question was sampled or recorded). Treatments: SILc = Silage + 6 kg concentrate pellets; LATc = Fresh grass harvested after 5 weeks regrowth + 6 kg concentrate pellets; LAT = Fresh grass harvested after 5 weeks regrowth; ERL = Fresh grass harvested after 3 weeks regrowth; all treatments were supplemented 0.6 kg/d of marker pellets.

5.3 Paper III

Shredding of grass-clover before ensiling: Effects on feed intake, digestibility, and methane production in dairy cows

Hansen, N.P.,[1] T. Kristensen,[2] M. Johansen,[1] A.L.F. Hellwing, [1] P. Waldemar,[3] and M.R. Weisbjerg[1].

[1]Department of Animal Science, AU Foulum, Aarhus University, 8830 Tjele, Denmark
[2]Department of Agroecology, AU Foulum, Aarhus University, 8830 Tjele, Denmark
[3]Kverneland Group Kerteminde A/S, Taarupstrandvej 25, 5300 Kerteminde, Denmark

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Shredding of grass-clover before ensiling: Effects on feed intake, digestibility, and methane production in dairy cows

Nikolaj Peder Hansen [a,*], Troels Kristensen [b], Marianne Johansen [a], Anne Louise Frydendahl Hellwing [a], Peter Waldemar [c], Martin Riis Weisbjerg [a]

[a] Department of Animal Science, Aarhus University, AU Foulum, Blichers Allé 20, DK-8830 Tjele, Denmark
[b] Department of Agroecology, Aarhus University, AU Foulum, Blichers Allé 20, DK-8830 Tjele, Denmark
[c] Kverneland Group Kerteminde A/S, Taarupstrandvej 25, DK-5300 Kerteminde, Denmark

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ABSTRACT

Shredding is a type of processing that mechanically treats forage in order to separate plant tissues apart and rupture the plant cell. This experiment aimed at investigating the effects of shredding grass-clover harvested at two developmental stages on feed intake, digestibility, and gas production. The grass-clover was harvested either at an early (May 14; ERL) or a late (May 29; LAT) developmental stage. Within each developmental stage, grass-clover was mown, wilted, and either baled and wrapped (CON) or shredded, baled, and wrapped (SHR). The four combinations of ensiled grass-clover (ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR) was fed to four rumen, duodenum, and ileum cannulated primiparous Danish Holstein cows in a 4 × 4 Latin square design with four periods of 21 d duration. The silage was offered for ad libitum intake. Silage density was higher ($P < 0.01$) for SHR compared to CON. Silage concentrations of L-lactate and acetate were higher ($P = 0.01$ and $P < 0.01$, respectively) and the silage concentration of butyrate was lower ($P < 0.01$) for SHR compared to CON, whereas pH was lower ($P = 0.02$), which indicated silage quality improved from shredding. The dry matter (DM) intake (DMI), milk yield, and rumen digestibility of neutral detergent fibre (aNDFom) were not affected by shredding, whereas feeding SHR compared to CON resulted in lower ($P = 0.05$) total tract digestibility of aNDFom (714 vs. 727 g/kg) and lower ($P = 0.04$) methane (CH_4) production (60 vs. 66 L CH_4/kg organic matter (OM) digested in the rumen). Compared to CON, SHR had a higher ($P = 0.02$) proportion of butyrate in rumen fluid indicating that shredding had some effect on the dynamics of rumen fermentation. However, the effective degradability of CP and aNDFom in the rumen determined in situ showed no effect of shredding. Rumination and total chewing time were lower ($P = 0.03$ and $P = 0.05$, respectively) and the concentration of protein in the milk was higher ($P = 0.02$), when shredding LAT compared to ERL. Furthermore, concentrations of CP and purines in rumen microbes were lower ($P = 0.04$ and $P = 0.01$, respectively), when cows were fed LAT

Abbreviations: AA, amino acids; ADF, acid detergent fibre; ADL, acid detergent lignin; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; CH_4, methane; CO_2, carbon dioxide; CON, control grass-clover; CP, crude protein; daNDFom, digested aNDFom; DM, dry matter; DMI, DM intake; dOM, digested organic matter; dRUP, digestibility of rumen-undegraded protein; ECM, energy corrected milk; EPD, effective protein degradability in the rumen; EPD_cor, EPD corrected for particle loss; ERL, grass-clover harvested at an early developmental stage; H_2, hydrogen; iNDF, indigestible aNDFom; LAT, grass-clover harvested at a late developmental stage; N, nitrogen; N_2, dinitrogen; NH_3-N, ammonia-N; O_2, oxygen; OM, organic matter; RQ, respiration quotient; RUP, rumen-undegraded protein; SEM, standard error of the mean; SHR, shredded grass-clover; TPD, total tract protein digestibility; VFA, volatile fatty acids.

* Corresponding author at: Department of Animal Science, Aarhus University, AU Foulum, Blichers Allé 20, DK-8830 Tjele, Denmark.
E-mail address: nikolaj.hansen@anis.au.dk (N.P. Hansen).

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compared to ERL. In conclusion, shredding improved silage fermentation quality, reduced digestibility of aNDFom without affecting the digestibility of other nutrients, and reduced methane production per kg of rumen digested OM. Furthermore, we observed no benefits from shredding grass-clover harvested at a late compared to early developmental stage, except for increased concentration of protein in milk and reduced time spent for chewing during rumination and total chewing.

1. Introduction

Grass-clover is an important feedstuff for dairy cows in Northern Europe. Farmers can increase their forage dry matter (DM) yields in the fields by harvesting the grass-clover at a later developmental stage. However, as the grass and legume plants develop and yields increase, specific plant tissues and specific plant cells become lignified resulting in a rapidly decreasing digestibility of the grass-clover (Jung et al., 2012).

Shredding, a type of processing (Koegel et al., 1988), where the particles of grass-clover are crushed and broken between two or more rollers rotating with different speed, has been investigated earlier with respect to effects on forage quality (Koegel et al., 1992; Descoteaux and Savoie, 2002). Compared to the traditional precision chopping, shredding attempts to disrupt the forage particles by separating rigid plant tissues apart and by rupturing a larger proportion of the plant cell walls, in order to increase the surface area for microbial adhesion and thereby increase rate of digestion (Lehmann et al., 2017). Indications of such effects were shown in an experiment, where we found that shredding increased silage density of grass and legumes (Samarasinghe et al., 2019). Shredding has shown varying effect on digestibility of both grasses and legumes (Broderick et al., 1999, 2002; Weisbjerg et al., 2018). This variation might be attributed to several factors that affect the dynamics between rumen fill and rate of degradation and passage of fibres in the rumen. However, more knowledge regarding effects of shredding grass-clover on these dynamics and especially degradation and passage kinetics in the rumen is required. By combining harvest of the grass-clover at a later developmental stage with shredding, farmers can potentially increase grass-clover DM yields without compromising the quality.

In physically processed grass-clover, fibre-bound proteins might be more prone to microbial digestion as shown in pulp by Damborg et al. (2018). As an effect of altering the rumen degradability of feed protein, the flow of amino acids (AA) in duodenum and the origin of the AA (i.e. feed, microbial, or endogenous) might be affected (Schwab and Broderick, 2017). Furthermore, shredding grass-clover has shown to decrease the time cows spend chewing (Weisbjerg et al., 2018), which in turn might affect the rumen environment (Beauchemin, 2018) and therefore affect methane (CH_4) production. Alongside with the possible effects on fibre digestibility, effects on protein digestibility, rumen environment, and CH_4 production are also to be considered if shredding is introduced in the farm management.

The aim of the current experiment was therefore to investigate the effects of using a machine for shredding of grass-clover harvested at an early and a late developmental stage on the degradation kinetics of especially fibre and protein and to evaluate the associated effects on feed intake and CH_4 production. We hypothesised that shredding of grass-clover could increase fibre digestibility and thereby feed intake. We also hypothesised that shredding grass-clover cut at late developmental stage would increase fibre digestibility more compared to shredding grass-clover cut at early developmental stage.

2. Materials and methods

The experiment complied with the guidelines set out by the Danish Ministry of Environment and Food (2014) Law No. 474 (May 15, 2014) concerning animal experiments and care of animals used for scientific purposes.

2.1. Forage production

A field with a mixture of perennial ryegrass (Lolium perenne L. 'Abosan', 'Diwan', and 'Humbi'), festulolium (Festulolium pabulare 'Fojtan'), white clover (Trifolium repens L. 'Silvester'), and red clover (Trifolium pratense L. 'Callisto') was established near Viborg, Denmark (56°31N 9°29E) in spring 2017. For every 100 g of the seed mixture, 45, 40, 6, and 9 g consisted of perennial ryegrass, festulolium, white clover, and red clover, respectively. In the spring growth of 2018, the field was divided in two and one half was harvested at an early (May 14; ERL) and the other half at a late (May 29, LAT) developmental stage. The grass-clover was mown using a disc mower without conditioner (Kuhn FC 883, Saverne, France) set at seven cm stubble height. The DM concentration was planned to be 350 g/kg; therefore, the grass-clover was wilted for one to two days before raking. At each harvest time, six samples of grass-clover were obtained before mowing and pooled to determine the clover and stem proportion (both on DM basis; 60 °C for 48 h in air-forced oven). In addition, the developmental stage of ryegrass, festulolium, red clover, and white clover was determined in the field before mowing according to the methods of Moore et al. (1991) and Skinner and Moore (2007).

At each harvest time, half of the grass-clover was baled and wrapped (McHale Fusion 3, Ballinrobe, Ireland) and constituted the control (CON). The chamber size in the baler was fixed (width = 1.23 m, diameter = 1.25 m, and volume = 1.51 m^3). The other half was shredded (SRH) in the field using a tractor-driven machine (Kverneland group A/S, Kerteminde, Denmark; Fig. 1). The machine was equipped with a pick-up that continuously channelled grass-clover into a space between a high-speed rotating drum (700 mm diameter and 1500 mm width) and a curved shell, which covered approximately half of the drum's circumference. The drum and the

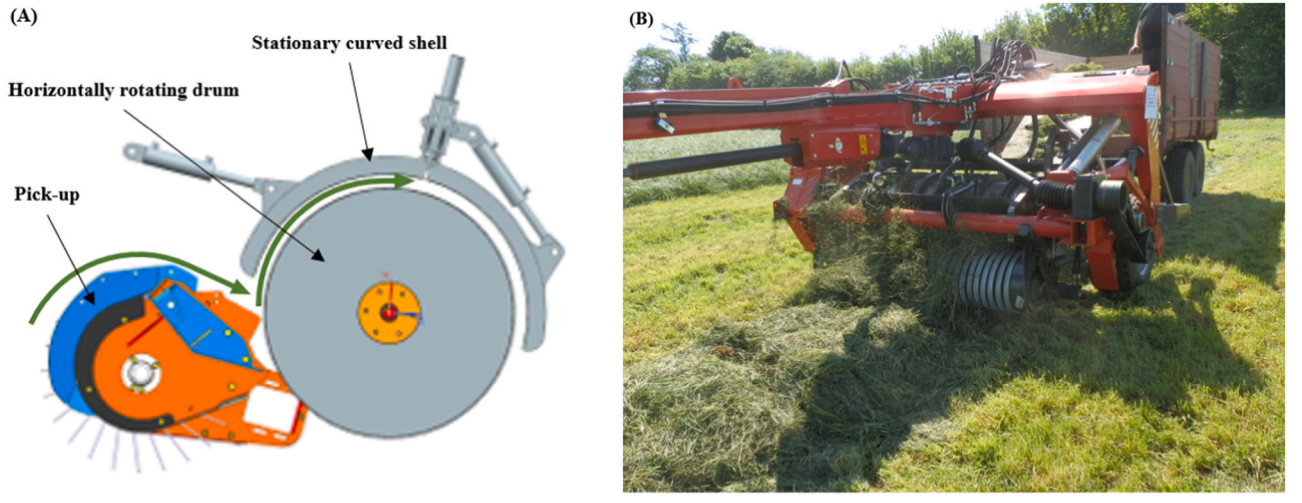

Fig. 1. Schematic diagram of the machine for shredding viewed from the side, where the curved arrows represent the flow of grass-clover (A; diagram by P. Waldemar), and a picture of the shredder prototype in operation (B; picture by M. R. Weisbjerg).

shell were equipped with 12 mm high oppositely oriented steel ridges (22 and 12, respectively). As the drum rotated (800 revolutions per min) and the shell was adjusted to create only a narrow funnel-shaped clearance through which the grass-clover could pass, the grass-clover was subjected to a physical treatment termed shredding. After shredding, the grass-clover was transported to a concrete bunker silo and placed in rows from which it was baled and wrapped using the same baler as used for CON. In total, four silages were produced differing in developmental stage and type of processing: grass-clover harvested at early developmental stage with or without shredding (ERL-SHR and ERL-CON, respectively) and at late developmental stage with or without shredding (LAT-SHR and LAT-CON, respectively). After each harvest time, all bales were transported to AU-Foulum, Aarhus University, where they were weighed at arrival.

2.2. Animals and feeding

Four primiparous Danish Holstein cows fitted with a cannula in the rumen (#1C, Bar Diamond Inc., Idaho, USA) and simple T-shaped cannulas (25 mm in diameter) in the proximal duodenum (approximately 50 cm caudal to pylorus) and terminal ileum were used in the experiment. At the beginning of the experiment, cows were on average (mean ± SD) 325 ± 57 days in milk, yielded 26.0 ± 3.8 kg milk/d, and weighed 565 ± 42.4 kg. The cows were housed in a tie-stall in cubicles with rubber mats and sawdust as bedding material. The cows were randomly assigned to one of four dietary treatments in a 4 × 4 Latin square design with four periods, each period lasting for 21 d. The cows were fed individually twice daily at 06.15 and 16.30 h for ad libitum intake of silages. Minerals (100 g; VM2 grøn, Vilofoss, Fredericia, Denmark) were offered daily and vitamins (20 g; Rød Suplex Caps, Vilofoss) were offered twice a week; both administered on top of silages during morning feeding. For one week before the experiment started, cows were gradually adapted to eat only grass-clover silage (spring growth; 202 g CP/kg DM and 328 g aNDFom/kg DM) without concentrate supplementation. The cows had free access to water. All cows were milked twice daily at 05.30 and 16.00 h. Digesta flow markers, 10 g chromium(III) oxide and 13 g titanium(IV) dioxide, weighed out in degradable filter paper bags, were placed manually in the rumen through the rumen cannula during each milking on d 0–16 in each period.

2.3. Sampling and recording

In each period, samples of silages and feed residues were collected daily from d 11–15 and from d 12–16, respectively for DM determination (air forced oven at 60 °C for 48 h), and samples for further analyses were stored at −20 °C. Furthermore, silages and residues were collected on d 17–20 and d 18–22, respectively, for DM determination. After the experiment ended, thawed samples of silages were first pooled within period (used for analysis of extracts; n = 4) and these samples were pooled again to obtain one sample from period 1 and 2 and one sample from period 3 and 4, which were stored at −20 °C before chemical and in situ analysis (n = 2).

Twelve subsamples of duodenal and ileal digesta as well as faeces were collected during a 94 h period from d 12–16 in each period (d 12: 10.00 h, 18.00 h; d 13: 02.00 h, 12.00 h, 20.00 h; d 14: 04.00 h, 14.00 h, 22.00 h; d 15: 06.00 h, 16.00 h, 24.00 h; d 16: 08.00 h). Duodenal (400 mL) and ileal (200 mL) digesta were collected by attaching a plastic tube to the cannulas. Faeces (300 mL) were collected when the cows defecated or by grab sampling after stimulation. The samples were pooled within cow and period and stored at −20 °C until chemical analysis. At the same 12 time points, rumen fluid was collected from the ventral rumen sac via the rumen cannula using a plastic syringe mounted to a strainer (#RT, Bar Diamond Inc.). The rumen fluid pH was measured immediately after sampling, and samples were stored at −20 °C until chemical analysis.

Chewing time was recorded simultaneously with digesta sampling from d 12–15 in each period using the RumiWatch system (ITIN+HOCH GmbH, Liestal, Switzerland). The cows were habituated to the halters from d 5–9 in each period. Raw data was converted using RumiWatch Converter 0.7.3.2, where the output data was selected for one hour resolution.

On d 16 in each period, rumen evacuations were performed on two cows at 12.00 h and on the remaining two cows at 12.30 h as described by Tothi et al. (2003). Composited samples made proportionally from the liquid and solid fractions were either used for DM determination (air forced oven at 60 °C for 48 h) or stored at −20 °C until chemical analysis. Two litres of rumen liquid were collected during the evacuation procedure and microbes in the liquid were purified following the procedure described by Johansen et al. (2017).

On d 16 after the afternoon milking, all cows were moved to individual 17 m^3 open-circuit transparent respiration chambers (Hellwing et al., 2012). Chambers were opened twice daily for milking, feeding, and cleaning, and it took on average 15 min per chamber. The feed was covered with a lid, which was removed automatically 30 min after doors were closed.

Cows acclimatised for one day and gas production was measured for four consecutive days starting on d 17. After two days of measurements, cows swapped champers diagonally before the last two days of measurements. The cows were fed and milked and gas production was quantified following the procedure described by Børsting et al. (2019), except that the concentration of CH_4 was measured using an infrared sensor (VIA-510, Horiba instruments, Kyoto, Japan), the concentration of oxygen (O_2) with a paramagnetic sensor (Columbus Instruments International, Columbus, USA), and the concentration of hydrogen (H_2) using an electrochemical sensor (City Technology LTD, Hampshire, UK). Furthermore, the gas sensors were calibrated on d 16 and 19 in each period using a span gas (Hellwing et al., 2012).

Recovery tests were performed both before, in between periods, and after the experiment for individual chamber correction. In total, 83 carbon dioxide (CO_2) recovery tests with a recovery of 98.8 ± 1.4% and 34 CH_4 recovery tests with a recovery of 99.6 ± 1.9% were performed. For O_2 and H_2, an average of the recovery of CO_2 and CH_4 was used. Gas production is reported in L under standard conditions (0 °C, 101.325 kPa).

Milk samples were collected from six consecutive milkings starting with the milking in the afternoon on d 12 and for two consecutive milkings during gas measurements starting with the milking in the afternoon on d 20.

2.4. Chemical analyses

Samples of silage, microbes, composited samples of rumen content, duodenal and ileal digesta, and faeces used for chemical analysis were freeze-dried and milled through a 1 mm screen (ZM 200 mill, Retch GmbH, Haan, Germany). Ash was determined by ignition at 525 °C for 6 h. The Dumas principle (Hansen, 1989) was used to measure total nitrogen (N) (Vario Max CN, Elementar Analysesysteme GmbH, Hanau, Germany) and CP was calculated as total N × 6.25. The concentration of neutral detergent fibre (aNDFom), acid detergent fibre (ADF), and acid detergent lignin (ADL) was determined sequentially following the Ankom procedures in an ANKOM 220 Fiber analyser (ANKOM Technology, Macedon, USA) using heat-stable α-amylase and sodium sulphite (Mertens, 2002) with correction for ash using the ash residue after the ADL procedure. Titanium(IV) dioxide was determined as described by Myers et al. (2004) with the modification that 15 instead of 10 mL of 30% hydrogen peroxide were added and five additional drops of hydrogen peroxide were added before measuring the absorbance. Chromium(III) oxide was analysed by spectrophotometry after oxidation with sodium peroxide to chromate (Schürch et al., 1950). Purines were analysed by spectrophotometry according to Zinn and Owens (1986) and modified by Thode (1999). In silages, soluble N was analysed by extraction in a 39 °C borate-phosphate buffer at pH 6.75 for 1 h. Total reducing sugars were estimated on glucose basis by the Luff-Schoorl method (Schoorl, 1929) as described in European Community (2012). Crude fat was determined by hydrolysis in HCl followed by extraction in petroleum ether (Stoldt, 1952), and non-fibre carbohydrates (NFC) was calculated as 1000 – (aNDFom + crude fat + CP + ash). *In vitro* OM digestibility using rumen fluid was determined according to Tilley and Terry (1963). Silage OM digestibility was calculated as 4.10 + 0.959× in vitro digestibility of OM according to Åkerlind et al. (2011).

In situ rumen degradation kinetics of aNDFom and CP were determined by incubating silage samples (1.5 mm milling; Dacron bags with 38 μm pore size) in the rumen of three non-lactating cows fed at maintenance level following the NorFor procedure (Åkerlind et al., 2011). Parameters for the degradation profile of CP were estimated according to Ørskov and McDonald (1979) and particle loss was estimated as the difference between solubility at 0 h and solubility over filter paper (Hvelplund and Weisbjerg, 2000). Effective protein degradability corrected for particle loss (EPD_cor) was calculated using a fractional rate of passage out of the rumen of 0.05 h^{-1}. Parameters for the degradation profile of aNDFom were estimated according to McDonald (1981) and feed residues after incubation were related to residues after 0 h incubation to account for possible particle losses. Effective degradability of aNDFom in the rumen was calculated using the fractional rate of passage determined for each silage based on rumen evacuations. The concentration of indigestible aNDFom (iNDF) in silage was determined as the aNDFom residue after rumen incubation of milled (1.5 mm cutter mill) samples placed in Dacron bags (12 μm pore size) in three non-lactating cows for 288 h (Åkerlind et al., 2011). Total tract protein digestibility (TPD) of silages was determined by the mobile bag technique (Hvelplund et al., 1992). Residues of both rumen and mobile bag incubations were transferred quantitatively to N-free filter paper, dried at 60 °C to determine DM, and analysed for N by the Kjeldahl method, while aNDFom residues were transferred quantitatively to filter crucibles and analysed using the Fibertech™ M6 System (Foss Analytical).

Silage extracts were produced as described by Johansen et al. (2017), except that 25% meta-phosphoric acid was used for stabilisation. According to Kristensen et al. (1996), total and individual VFA in rumen fluid and silage extracts were analysed using GC. Ammonia-N (NH_3-N) was analysed on a Cobas Mira autoanalyser (Triolab A/S, Brøndby, Denmark). L-lactate and glucose were analysed using the immobilised glucose oxidase electrode technique (Mason, 1983; YSI 2900D, YSI Inc., Yellow Springs, USA). Crude protein, fat, and lactose monohydrate in milk were analysed using a Milkoscan 4000 analyser (Foss Analytical, Hillerød, Denmark) at Eurofins Steins (Vejen, Denmark).

2.5. Calculations

Silage density was calculated as the individual bale weight divided by the chamber size (1.51 m^3). The concentration of net energy for lactation (NEL) was determined using NorFor (Volden, 2011). Intake of DM (DMI) was calculated as DM offered minus DM in the residue and averaged across d 11–15 and across d 17–20 in each period per cow. Average milk yield was determined across d 11–15 and across d 17–20 in each period. Daily weight change of cows were calculated as the weight on d 21 minus weight on d −1 divided by 21. Assuming that the pooled sample reflected the true flow, flow of DM was determined by each marker, the average hereof was used for DM and nutrient flows reported, and for calculation of digestibility. Rumen digestibility of aNDFom was calculated as aNDFom intake minus flow in ileum divided by intake, since calculations based on duodenal flow of aNDFom have yielded unreasonable results (Brask et al., 2013). True ruminal digestibility of DM, OM, and CP was calculated by correcting for microbial flow in the duodenum. Digestibility of RUP (dRUP) was calculated as: dRUP (g/kg CP) = [TPD (g/kg CP)–EPD_cor (g/kg CP)]/[1000–EPD_cor (g/kg CP)] × 1000 (Hvelplund and Weisbjerg, 2000).

Energy corrected milk (ECM, 3.14 MJ/kg) yield was calculated as: ECM = milk yield (kg) × [(38.3 × fat (g/kg) + 24.2 × protein (g/kg) + 15.71 × lactose (g/kg) + 20.7)/3140], where lactose is lactose monohydrate (Sjaunja et al., 1990). Methane production was related to DMI and ECM yield measured during the gas measurements, and related to aNDFom and OM digested in the rumen by multiplying the DMI measured during the gas measurements by the rumen digestibility of aNDFom and OM estimated during the digesta samplings. Variables for rumen liquid (pH, total VFA, VFA proportions, L-lactate, NH_3-N, and glucose) were averaged into a daily mean per cow per period before the statistical analysis.

2.6. Statistical analyses

All statistical analyses were performed in R 4.0.4 (R Core Team, 2021). The effect of silage type was analysed using a linear mixed

effects model with the lmer function from the lme4 package (Bates et al., 2015):

$$Y_{pdtc} = \mu + \alpha_p + \beta_d + (\alpha\beta)_{pd} + \tau_t + A_c + \varepsilon_{pdtc},$$

where Y_{pdtc} is the dependent response variable, μ is the overall mean, α is the fixed effect of processing (p = CON, SHR), β is the fixed effect of developmental stage (d = ERL, LAT), $(\alpha\beta)_{pd}$ is the interaction between processing and developmental stage, τ is the fixed effect of period (t = 1–4), A is the random effect of cow (c = 1–4), and ε_{pdtc} is the random residual error assumed to be normal distributed and independent with constant variance. When interactions between developmental stage and processing were significant, pairwise comparisons were analysed using Tukey's test. Data for H_2 production was log transformed to obtain variance homogeneity. The transformed least square means, associated *P*-values, and least square means from model without transformation are shown in the table. The fixed effect of period and random effect of cow were excluded when analysing the response variables related to chemical composition of the silages and the in situ degradation characteristics using the lm function in R 4.0.4 (R Core Team, 2021).

Digesta from ileum from one cow in period three and four receiving ERL-CON and ERL-SHR, respectively, was lost due to cannula problems. Data for chewing time from the cow fed LAT-SHR in period one was lost due to technical breakdown. Mean values shown in the tables are least squares means with the highest corresponding standard error of mean. Statistical significance was considered as *P*-values ≤ 0.05 and tendencies as $0.05 < P\text{-values} \leq 0.10$.

3. Results

Results on AA composition in silages and rumen microbes as well as total duodenal flow of individual AA are reported in Supplementary Tables S1 and S2.

3.1. Silages

The 15 day difference between ERL and LAT resulted in a 68% higher stem proportion and more mature plants for LAT compared to ERL (Table 1). Across developmental stage, bale weight and density were 30% greater ($P < 0.01$) for SHR compared to CON (Table 2). The concentration of aNDFom and iNDF was 29% and 125% higher ($P < 0.01$), respectively, in LAT compared to ERL, and the in vitro OM digestibility was lower ($P < 0.01$) in LAT compared to ERL (641 vs. 776 g/kg, respectively). Compared to CON, SHR decreased ($P = 0.02$) silage pH with 0.28 units and decreased ($P < 0.01$) the concentration of butyrate by 2.22 g/kg DM, while increasing the concentration of acetate ($P < 0.01$) and L-lactate ($P = 0.01$) by 2.59 and 7.73 g/kg DM, respectively. Shredding of LAT increased the concentration of insoluble, but rumen-degradable fraction of CP compared to shredding of ERL ($P = 0.03$ for interaction; Table 3).

3.2. Feed intake, digestibility, chemical composition of microbes, and rumen pools

The DMI averaged 9.8 kg/d (Table 4), but no differences between SHR and CON were observed. Shredding increased intake of aNDFom for LAT, but decreased for ERL ($P = 0.04$ for interaction). The duodenal flow of total AA was lower ($P = 0.01$) in cows fed LAT compared to ERL (1092 vs. 1500 g/d, respectively). Rumen digestibility of aNDFom was not affected by shredding, whereas total tract digestibility was lower ($P = 0.05$) for SHR compared to CON (714 vs 728 g/kg, respectively).

Shredding had no effect on the chemical composition of microbes or the efficiency of microbial protein synthesis (Table 5). However, when cows were fed LAT compared to ERL, concentrations of CP (497 vs. 534 g/kg DM, respectively) and purines (91.3 vs. 102 g/kg DM, respectively) in microbes were lower ($P = 0.04$ and $P = 0.01$, respectively).

Overall, average rumen pool size of DM was 7.9 kg, and there was no difference in rumen pool size or the rates of digestion and passage out of the rumen between SHR and CON (Table 6). When cows were fed LAT compared to ERL, rumen pool size of aNDFom and iNDF was 28% and 84% higher ($P < 0.01$), respectively.

Table 1
Stem proportion, botanical composition, and developmental stage of grass-clover harvested at two developmental stages[1].

Development stage	Early	Late
Stem proportion, g/kg DM	336	564
Botanical composition		
White clover, g/kg DM	5.51	16.5
Red clover, g/kg DM	74.4	77.6
Grass, g/kg DM	920	904
Developmental stage[2]		
Ryegrass	Elongation stage 1	Reproductive stage 1
Festulolium	Elongation stage 2	Reproductive stage 3
Red clover	Mid vegetative = 1, 16–30 cm	Late bud = 4–5
White clover	Vegetative = 0, only leaves	Vegetative = 0, only leaves

[1] The developmental stages correspond to the two treatments ERL and LAT.
[2] Developmental stage was determined according to Moore et al. (1991) and Skinner and Moore (2007).

Table 2
Density and chemical composition of grass-clover silages[1] (n = 16 for DM, n = 4 for extracts, NH_3-N, and pH, and n = 2 for all other variables).

Development stage	Early		Late		SEM	*P*–value[2]		
Processing	Control	Shredded	Control	Shredded		D	P	D×P
Bale weight[3], kg	689	860	630	856	25.0	0.03	< 0.01	0.19
Density[4], kg/m^3	457	570	417	567	16.6	0.03	< 0.01	0.19
DM, g/kg FM	499	530	378	401	0.6	< 0.01	< 0.01	0.50
Ash, g/kg DM	83.4	87.3	68.9	64.9	2.58	< 0.01	0.97	0.20
CP, g/kg DM	188^a	197^a	134^b	130^b	2.2	< 0.01	0.28	0.04
Soluble N, g/kg N	652	612	701	673	9.9	0.01	0.03	0.55
NH_3-N, g/kg N	44.9^c	47.7^c	78.7^a	65.5^b	2.51	< 0.01	0.06	0.01
AA-N[5], g/kg N	642^a	622^a	575^c	597^b	3.8	< 0.01	0.84	< 0.01
Crude fat	35.0	34.5	26.0	26.0	1.82	0.01	0.90	0.90
Sugar, g/kg DM	112	92.2	43.0	38.8	6.90	< 0.01	0.16	0.33
aNDFom, g/kg DM	446	434	567	565	5.7	< 0.01	0.28	0.44
iNDF[6], g/kg aNDFom	95.5	105	223	228	3.05	< 0.01	0.07	0.56
ADF, g/kg DM	252	249	330	331	2.9	< 0.01	0.77	0.54
ADL, g/kg DM	13.0	12.5	26.2	24.0	1.29	< 0.01	0.35	0.54
NFC[7]	215	233	176	184	7.6	< 0.01	0.16	0.55
OMD[8], g/kg OM	774	778	637	645	4.3	< 0.01	0.20	0.62
NEL[9], MJ/kg DM	6.39	6.36	5.18	5.26	0.034	< 0.01	0.46	0.16
Total AA, g/kg DM	143	146	92.2	93.4	1.26	< 0.01	0.16	0.51
Extracts, g/kg DM								
pH	5.32	5.05	4.56	4.27	0.107	< 0.01	0.02	0.91
Acetate	13.4	15.9	19.5	22.2	0.67	< 0.01	< 0.01	0.93
Propionate	0.848^b	0.000^b	4.17^a	0.000^b	0.4350	< 0.01	< 0.01	< 0.01
Butyrate	1.60	0.000	3.53	0.680	0.4550	0.01	< 0.01	0.19
Isovalerate	0.000	0.0972	0.000	0.120	0.07724	0.88	0.18	0.88
Caproate	0.298	0.287	0.383	0.375	0.0107	< 0.01	0.40	0.92
Glucose	42.4	35.7	19.2	17.1	4.18	< 0.01	0.31	0.59
L-lactate[10]	21.9	31.9	32.2	37.7	2.44	0.01	0.01	0.37

[abc]Values within the same line with different superscripts differ ($P \leq 0.05$).
[1] The combinations of developmental stage and processing correspond to the four treatments ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR.
[2] D=developmental stage; P = processing.
[3] n = 12, 6, 12, and 5 for ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR, respectively.
[4] Based on fixed volume in baler (width = 1.23 m; diameter = 1.25 m; volume = 1.51 m^3).
[5] Amino acid nitrogen.
[6] Indigestible aNDFom; Dacron bags were incubated into three non-lactating cows (Åkerlind et al., 2011)
[7] Non-fibre carbohydrate calculated as 1000−(aNDFom + crude fat + CP + ash).
[8] *In vivo* organic matter digestibility calculated as 4.10 + 0.959× in vitro digestibility of OM according to Åkerlind et al. (2011).
[9] NEL_{20}, net energy for lactation, calculated in NorFor according to Volden (2011).
[10] L-lactate constitutes about half of total lactate (Johansen et al., 2020).

3.3. Milk and gas production

There was no overall difference in milk production between CON and SHR (Table 7). The concentration of protein in milk was lower ($P = 0.01$) for LAT-CON compared to ERL-CON, but no difference was found between developmental stages when the grass-clover was shredded. Across treatments, body weight decreased ($P = 0.04$; ANOVA) 40 kg from the beginning to the end of the experiment, and daily weight change was larger ($P = 0.03$) for cows fed LAT compared to cows fed ERL.

Overall, CH_4 averaged 329 L/d (Table 8). If expressed as L/kg OM digested in the rumen, the production of CH_4 was lower ($P = 0.04$) when cows were fed SHR compared to CON (59.2 vs. 65.6 L/kg OM digested in the rumen, respectively).

3.4. Rumen fluid

Compared to CON, rumen fluid concentration of butyrate (10.5 vs. 9.47 mol/100 mol of total VFA, respectively) and caproate (0.633 v. 0.770 mol/100 mol of total VFA, respectively) were higher ($P = 0.02$) and lower ($P = 0.02$), respectively, when cows were fed SHR (Table 9). Furthermore, when LAT was shredded, the concentration of NH_3-N was lower ($P = 0.05$) compared to shredding of ERL. When cows were fed LAT compared to ERL rumen fluid pH tended to be higher (6.71 vs. 6.60, respectively; $P = 0.09$), whereas the concentration of total VFA was 11 mmol/L lower ($P < 0.01$), and the proportion of especially propionate was higher (19.4 vs. 18.5 mol/100 mol of total VFA; $P < 0.01$).

3.5. Chewing time

When expressed as min/kg DMI, cows fed LAT compared to ERL spent 18 min more ($P < 0.01$) for rumination and 22 min more ($P = 0.02$) on total chewing (Table 10). An interaction between developmental stage and processing showed that rumination time

Table 3
In situ parameter estimates (a, b, and c) and degradation characteristics[1] of CP and aNDFom of grass-clover silages[2] determined using non-lactating and lactating cows[3] (n = 2).

Development stage	Early		Late		SEM	*P*–value[4]		
Processing	Control	Shredded	Control	Shredded		D	P	D×P
CP								
a, g/kg	676	681	764	722	9.6	< 0.01	0.13	0.07
b, g/kg	286[a]	281[a]	142[c]	191[b]	8.4	< 0.01	0.06	0.03
c, h^{-1}	0.120	0.115	0.0704	0.0857	0.00562	< 0.01	0.40	0.15
Particle loss g/kg	77.8	104	111	116	14.01	0.18	0.32	0.51
EPD_cor[5], g/kg	849	839	776	778	11.2	< 0.01	0.76	0.61
TPD, g/kg	910	915	862	868	3.6	< 0.01	0.21	0.90
dRUP, g/kg	402	468	382	400	27.3	0.18	0.19	0.42
aNDFom								
b, g/kg	892	880	765	761	4.4	< 0.01	0.13	0.46
c, h^{-1}	0.0608	0.0587	0.0408	0.0445	0.00303	< 0.01	0.81	0.39
Lag time, h	0.384	0.302	0.488	0.164	0.1945	0.94	0.35	0.57
ED_NDF[6], g/kg	707	694	520	526	7.2	< 0.01	0.67	0.28

[abc]Values within the same line with different superscripts differ ($P \leq 0.05$).

[1] a = rumen-soluble fraction; b = insoluble, but rumen-degradable fraction; c = fractional rate of degradation of fraction b; EPD_cor = effective protein degradability in the rumen corrected for particle loss; TPD = true protein degradability; dRUP = digestibility of rumen undegraded protein; ED_NDF = effective rumen degradability of aNDFom.

[2] The combinations of developmental stage and processing correspond to the four treatments ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR.

[3] Dacron bags were incubated into three non-lactating cows (Åkerlind et al., 2011); TPD and dRUP determined by incubating Dacron bags in three non-lactating cows followed by inserting the same bags in duodenum of three lactating cows (Hvelplund et al., 1992).

[4] D=developmental stage; P = processing.

[5] Calculated using a fractional rate of passage of 0.05 h^{-1}.

[6] Calculated using the fractional rate of passage determined for each silage based on rumen evacuations (Table 6).

expressed as min/kg DMI was lower ($P < 0.01$) when cows were fed ERL-CON compared to LAT-CON, lower ($P = 0.02$) when cows were fed ERL-CON compared to LAT-SHR, and higher ($P < 0.01$) when they were fed LAT-CON compared to ERL-SHR. Furthermore, an interaction between developmental stage and processing showed that total chewing time expressed as min/kg DMI was higher ($P = 0.03$) for LAT-CON compared to ERL-CON, and that this difference between developmental stage disappeared, when the grass-clover was shredded.

4. Discussion

4.1. Silages

In this experiment, shredding affected several parameters related to silage quality. The achieved DM concentrations of the silages were higher than the intended 350 g/kg due to warm weather conditions during wilting and processing. However, there was only minor difference in DM concentration between CON and SHR within each developmental stage. A low pH and the concentration of fermentation products indicated that all silages were well preserved (McDonald et al., 1991). As in the current experiment, shredding prior to ensiling have shown to increase silage density of grass-clover (Samarasinghe et al., 2019) and lucerne (Shinners et al., 1988) in lab-scale experiments. In both cases, increased density was most likely caused by rupture of the rigid structure of stem tissues during shredding. In addition, shredding seemed not to cause any loss of effluent from the ensiled bales in the current experiment, which was probably due to the relatively high DM concentrations. In the current experiment, there was only limited visual difference between CON and SHR silages. However, shredding increased density of the bales and increased the concentration of L-lactate and acetate in silages, which lead to a subsequent reduction in pH. This indicated that the plant cells indeed were ruptured during shredding making more carbohydrates available for the ensiling process. Furthermore, formation of butyrate was avoided when the grass-clover was shredded, which improved the fermentation profile (McDonald et al., 1991). However, it was not possible to distinguish between the effects of increased density and the effects of shredding on the fermentation quality in the current experiment.

4.2. Feed intake and milk production

The achieved DMI in the current experiment reflected that the cows were in late lactation and that the cows were fed solely grass-clover silage with a relatively low concentration of NEL. Since cows in late lactation were used, we expected only limited effects on milk production. Although an interaction between developmental stage at harvest and shredding was statistically detected for milk protein concentration, the numeric difference was negligible. The cows were habituated to an only-forage diet prior to the experiment, which therefore only resulted in a minor numeric decrease in DMI from period 1 to period 4 (mean ± SD; 11.3 ± 3.56–9.2 ± 1.80 kg DMI/d, respectively).

Shredding of the grass-clover prior to ensiling had no effect on feed intake, which is in alignment with Weisbjerg et al. (2018) and

Table 4
Intake and in vivo digestibility in the rumen, small intestine, and total tract of four cows in a Latin square experiment fed grass-clover[1] silage (n = 4, except for apparent small and large intestinal digestibility, where n = 3 for ERL-CON and ERL-SHR).

Development stage	Early		Late		SEM	*P*–value[2]		
Processing	Control	Shredded	Control	Shredded		D	P	D×P
Intake, kg/d								
DM	11.7	10.6	7.98	9.10	1.186	< 0.01	0.97	0.12
OM	10.7	9.68	7.43	8.50	1.088	0.01	0.96	0.10
CP	2.18	2.08	1.07	1.18	0.219	< 0.01	0.95	0.46
Crude fat	0.350	0.326	0.277	0.275	0.0383	0.09	0.69	0.72
AA	1.67	1.55	0.736	0.851	0.1647	< 0.01	0.99	0.29
aNDFom	5.20[a]	4.60[a]	4.53[a]	5.13[a]	0.531	0.77	0.99	0.04
ADF	2.94[a]	2.64[a]	2.63[a]	3.00[a]	0.307	0.84	0.81	0.04
ADL	0.153	0.133	0.210	0.219	0.0195	< 0.01	0.62	0.21
NFC	2.96	2.68	1.56	1.92	0.314	< 0.01	0.81	0.07
Total flow in duodenum, kg/d								
DM	7.85	6.86	6.19	6.62	6.22	0.03	0.44	0.08
CP	2.27	2.00	1.52	1.58	1.90	< 0.01	0.43	0.24
Total AA	1.59	1.41	1.06	1.12	1.44	0.01	0.54	0.26
Apparent rumen digestibility								
aNDFom, g/kg	779	769	642	631	11.2	< 0.01	0.24	0.95
True rumen digestibility								
DM, g/kg	455	457	331	373	39.4	0.01	0.45	0.48
OM, g/kg	591	593	485	522	30.8	< 0.01	0.23	0.27
CP, g/kg	329	358	-47.8	76.9	95.33	< 0.01	0.25	0.46
AA, g/kg	426	433	-16.3	143	121.20	< 0.01	0.35	0.39
Apparent small intestinal digestibility								
DM, g/kg	588	571	491	463	27.0	< 0.01	0.17	0.72
OM, g/kg	534	524	422	389	32.4	< 0.01	0.17	0.47
CP, g/kg	706	700	741	724	20.9	0.12	0.42	0.73
Apparent total tract digestibility								
DM, g/kg	764	766	650	651	3.8	< 0.01	0.60	0.79
OM, g/kg	777	780	666	664	4.6	< 0.01	0.90	0.42
CP, g/kg	714	735	646	645	7.2	< 0.01	0.14	0.13
aNDFom, g/kg	802	797	653	631	9.5	< 0.01	0.05	0.16
ADF, g/kg	830	831	682	667	8.9	< 0.01	0.27	0.24

[a] Values within the same line with different superscripts differ (P ≤ 0.05).
[1] The combinations of developmental stage and processing correspond to the four treatments ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR.
[2] D=developmental stage; P = processing.

Table 5
Microbial nutrient composition and efficiency of microbial protein synthesis in four cows in a Latin square experiment fed grass-clover silage[1] (n = 4).

Development stage	Early		Late		SEM	*P*–value[2]		
Processing	Control	Shredded	Control	Shredded		D	P	D×P
Microbial composition, g/kg DM								
Ash	206	214	254	221	15.2	0.08	0.38	0.17
CP	538	531	481	512	18.6	0.04	0.46	0.23
Purines	102	101	87.7	94.8	4.51	0.01	0.31	0.13
AA	427	420	379	410	16.0	0.07	0.39	0.19
Efficiency OM[3]	161	148	148	134	8.4	0.12	0.12	0.97
Efficiency aNDFom[4]	198	205	149	152	9.6	< 0.01	0.56	0.81

[1] The combinations of developmental stage and processing correspond to the four treatments ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR.
[2] D = developmental stage; P = processing.
[3] Efficiency of microbial protein synthesis estimated as g microbial CP produced/kg OM digested in the rumen.
[4] Efficiency of microbial protein synthesis estimated as g microbial CP produced/kg aNDFom digested in the rumen.

Broderick et al. (2002). The in vivo determined fractional rate of degradation and passage of fibre were not affected by shredding in the current experiment, which could explain the lack of effect. Reducing the forage particle size with respect to the theoretical length of chopping have shown to both increase (Yang and Beauchemin, 2007) and reduce (Tayyab et al., 2018) DMI. However, reducing the theoretical length of chopping compared to shredding, as in the current experiment, are two different approaches to potentially increase utilisation of the forage, assuming that the feed particle surface differs between the two types of processing (Wilson and Kennedy, 1996). Shredding was believed to increase the fractional rate of degradation resulting in a reduced rumen fill value and a potentially increased feed intake. However, increased feed intake was not attained, indicating that a more intensive physical shredding should be tested in future research.

In the current experiment, we hypothesised that feed intake would increase, when cows were fed grass-clover harvested at an

Table 6
Composition of rumen content, rumen pool sizes, and rates of digestion and passage in four cows in a Latin square experiment fed grass-clover silage[1] (n = 4, except for kd_NDF, where n = 3 for ERL-CON and ERL-SHR).

Development stage	Early		Late		SEM	*P*–value[2]		
Processing	Control	Shredded	Control	Shredded		D	P	D × P
Rumen evacuation								
Total content, kg	74.0	73.6	78.5	81.1	6.78	0.17	0.79	0.71
Free fluid, kg	21.4	25.2	25.4	26.0	2.52	0.21	0.25	0.39
Fluid proportion, g/kg	295	343	326	314	23.8	0.95	0.29	0.11
Composition of rumen content								
DM, g/kg	107	104	99.7	103	3.41	0.13	0.98	0.19
aNDFom, g/kg DM	534	537	663	681	12.8	< 0.01	0.35	0.49
iNDF[3], g/kg DM	164	162	292	295	6.8	< 0.01	0.99	0.64
pdNDF[4], g/kg DM	370	375	370	386	17.9	0.68	0.45	0.72
Pool sizes								
DM, kg	7.96	7.65	7.81	8.29	0.693	0.58	0.84	0.38
aNDFom, kg	4.27	4.15	5.16	5.63	0.450	0.01	0.56	0.33
iNDF, kg	1.31	1.23	2.28	2.43	0.145	< 0.01	0.72	0.25
pdNDF, kg	2.96	2.92	2.88	3.20	0.334	0.65	0.53	0.42
Rates[5]								
kdNDF, h^{-1}	0.0567	0.0499	0.0423	0.0424	0.00323	0.01	0.39	0.30
kpiNDF, h^{-1}	0.0154	0.0150	0.0184	0.0197	0.00134	< 0.01	0.58	0.28

[1] The combinations of developmental stage and processing correspond to the four treatments ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR.
[2] D = developmental stage; P = processing.
[3] Indigestible aNDFom; Dacron bags were incubated into three non-lactating cows (Åkerlind et al., 2011).
[4] Potentially digestible aNDFom.
[5] kdNDF = fractional rate of degradation of aNDFom; kpiNDF = fractional rate of passage of iNDF out of the rumen.

Table 7
Milk yield, milk composition, and weight change in four cows in a Latin square experiment fed grass-clover silage[1] (n = 4).

Development stage	Early		Late		SEM	*P*–value[2]		
Processing	Control	Shredded	Control	Shredded		D	P	D×P
Milk yield, kg/d	12.2	12.4	8.99	9.25	2.016	< 0.01	0.74	0.96
ECM yield, kg/d	12.4	12.7	9.05	10.0	1.852	< 0.01	0.21	0.53
Milk composition								
Fat, g/kg	42.9	47.0	45.0	47.5	4.69	0.67	0.29	0.79
Protein, g/kg	36.0[a]	34.6[ab]	33.3[b]	34.5[ab]	1.34	0.02	0.81	0.02
Lactose, g/kg	44.0	42.8	43.3	43.4	1.14	0.94	0.53	0.43
DWC[3], g	-23.8	35.7	-1119	-774	373.00	0.03	0.60	0.71

[ab] Values within the same line with different superscripts differ (P ≤ 0.05).
[1] The combinations of developmental stage and processing correspond to the four treatments ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR.
[2] D = developmental stage; P = processing.
[3] Daily weight change.

earlier developmental stage or when grass-clover was shredded prior to ensiling. Since only silages were fed in the current experiment, DMI was assumed to be regulated physically (Rinne et al., 2002), which is attributed to the rumen pool size of aNDFom (Huhtanen et al., 2016). However, rumen pool size of aNDFom differed between ERL and LAT, whereas the pool size of DM did not. This indicated that the total content of DM in the rumen restricted further feed intake in cows fed LAT compared to ERL. Furthermore, the daily weight change for cows were low (ERL) and negative (LAT) and indicated that cows indeed were physically regulated. The DMI decreased 0.17 kg/day for every 10 g/kg decrease in aNDFom digestibility for LAT compared to ERL, which corresponded to findings by Oba and Allen (1999).

4.3. Digestibility

Generally, there was an unexpected lack of effect of shredding on many of the response variables in the current experiment. Total tract digestibility of aNDFom was reduced when grass-clover was shredded, contradicting findings by Weisbjerg et al. (2018). However, Weisbjerg et al. (2018) used a late summer cut clover rich grass-clover in regrowth, which might have differed in plant organ proportions (Søgaard, 2011) compared to the current experiment. Furthermore, Broderick et al. (2002) showed a decrease in total tract digestibility of ADF when shredding ryegrass, whereas shredding had no effect on total tract digestibility of ADF in the current experiment. Besides these studies, literature comparing in vivo digestibility of shredded and non-shredded grass is sparse. Other research have focused on shredding of lucerne or on the effect of theoretical length of chopping of both grass and lucerne (Kornfelt et al., 2013). Broderick et al. (1999) reported an increase in total tract digestibility of aNDFom when shredding lucerne, whereas

Table 8
Gas production from four cows in a Latin square experiment fed grass-clover silage[1] (n = 4).

Development stage	Early		Late		SEM	*P*–value[2]		
Processing	Control	Shredded	Control	Shredded		D	P	D×P
Gas production								
CH_4, L/d	385	347	293	290	31.8	< 0.01	0.25	0.32
CO_2, L/d	4834	4565	3654	3677	358.0	< 0.01	0.59	0.53
O_2, L/d	4684	4314	3707	3579	325.5	< 0.01	0.25	0.56
H_2, Log(L/d)	1.97	1.49	0.161	0.154	0.4287	< 0.01	0.46	0.47
H_2[3], L/d	7.87	7.23	1.41	1.33				
RQ[4]	1.0	1.1	0.987	1.03	0.0210	0.07	0.08	0.65
CH_4/CO_2	0.0796	0.0761	0.0798	0.0789	0.00296	0.17	0.06	0.23
CH_4 production								
L/kg DMI	32.5	29.6	31.6	30.5	1.67	1.00	0.13	0.45
L/kg dOM[5]	60.6	55.3	70.6	63.0	4.70	0.01	0.04	0.65
L/kg daNDFom[6]	88.1	85.1	86.8	85.7	4.70	0.94	0.67	0.84

[1] The combinations of developmental stage and processing correspond to the four treatments ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR.
[2] D = developmental stage; P = processing.
[3] LSM from model without log-transformation.
[4] Respiration quotient = CO_2 produced divided by the O_2 consumed.
[5] L CH_4/kg OM digested in the rumen.
[6] L CH_4/kg aNDFom digested in the rumen.

Table 9
Rumen pH and composition of rumen liquid from four cows in a Latin square experiment fed grass-clover silage[1] (n = 4).

Development stage	Early		Late		SEM	*P*–value[2]		
Processing	Control	Shredded	Control	Shredded		D	P	D×P
pH	6.62	6.57	6.70	6.71	0.069	0.09	0.74	0.65
Total VFA, mmol/L	114	114	103	103	6.2	< 0.01	0.99	0.96
VFA proportions, mol per 100 mol of total VFA								
Acetate	65.4	65.2	66.5	64.6	0.58	0.59	0.07	0.09
Propionate	18.3	18.7	19.0	19.7	0.38	0.02	0.11	0.62
Isobutyrate	1.25	1.21	1.17	1.12	0.069	0.16	0.45	0.96
Butyrate	10.1	10.5	8.84	10.4	0.369	0.08	0.02	0.10
Isovalerate	1.98	1.78	1.83	1.66	0.198	0.51	0.37	0.96
Valerate	2.17	2.09	1.88	1.83	0.116	0.03	0.49	0.86
Caproate	0.81^{a}	0.56^{b}	0.73^{ab}	0.71^{ab}	0.066	0.43	0.02	0.05
Acetate:propionate	3.59	3.56	3.53	3.32	0.096	0.07	0.14	0.27
L-lactate[3] mmol/L	0.270	0.265	0.0822	0.0872	0.09533	0.10	1.00	0.96
NH_3-N, mmol/L	9.28^{a}	9.41^{a}	6.94^{b}	5.77^{b}	0.459	< 0.01	0.10	0.05
Glucose, mmol/L	0.256	0.211	0.153	0.126	0.0383	< 0.01	0.03	0.48

[ab] Values within the same line with different superscripts differ (P ≤ 0.05).
[1] The combinations of developmental stage and processing correspond to the four treatments ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR.
[2] D = developmental stage; P = processing.
[3] L-lactate constitutes about half of total lactate (Johansen et al., 2020).

Table 10
Chewing time spent for eating and ruminating in four cows in a Latin square experiment fed grass-clover silage[1] (n = 4, except for LAT-SHR where n = 3).

Development stage	Early		Late		SEM	*P*–value[2]		
Processing	Control	Shredded	Control	Shredded		D	P	D×P
Min/day								
Eating	529	531	482	458	32.4	0.06	0.70	0.62
Rumination	518	482	543	561	44.7	0.21	0.74	0.47
Total chewing	1048	1013	1025	1017	36.3	0.69	0.44	0.64
Other activity	388	424	414	423	36.2	0.64	0.41	0.64
Min/kg DMI								
Eating	47.9	59.7	62.9	50.8	9.73	0.53	0.87	0.11
Rumination	45.1^{c}	50.6^{bc}	70.3^{a}	62.0^{ab}	4.33	< 0.01	0.76	0.03
Total chewing	93.0^{b}	110^{ab}	133^{a}	113^{ab}	12.8	0.02	0.94	0.05

[abc] Values within the same line with different superscripts differ (*P* ≤ 0.05).
[1] The combinations of developmental stage and processing correspond to the four treatments ERL-CON, ERL-SHR, LAT-CON, and LAT-SHR.
[2] D = developmental stage; P = processing.

shredding of grass-clover in the current experiment decreased total tract digestibility of aNDFom. For lucerne, reducing theoretical length of chopping resulted in a reduced total tract digestibility of aNDFom (Yang and Beauchemin, 2007), whereas reducing theoretical length of chopping in grass increased total tract digestibility of aNDFom (Tayyab et al., 2018; Haselmann et al., 2019). This emphasise that the effect of size and shape of green forage particles on fibre digestibility is complex and that other factors such as forage type contribute with varying effects.

Based on digestibility and rumen degradation kinetic experiments (Broderick et al., 1999; Weisbjerg et al., 2018), we hypothesised that shredding would increase exposure of cell walls and fibre bound proteins for rumen microbes resulting in faster degradation compared to the control silages. However, the lack of differences in EPD_cor between SHR and CON indicated that more intensive shredding was needed to attain this. Furthermore, the in vivo determined duodenal flow of total AA and the in situ determined intestinal digestibility of rumen-undegraded feed protein (dRUP) did not improve from shredding for neither ERL nor LAT. Shredding also had no effect on the duodenal flow of individual AA or the microbial composition of AA. The lack of difference in the in vivo determined kdNDF between SHR and CON supported that the intensity of shredding had not been sufficient. Here it must be emphasised that according to the small SEM achieved for treatment means, in vivo kdNDF was determined with a high level of precision. In the current experiment, stem proportion was higher for LAT compared to ERL, and shredding was therefore expected to improve NDF digestibility more for LAT compared to ERL. This was not attained since the intensity of shredding probably was not sufficient. However, the insoluble, but rumen-degradable fraction of CP determined in situ increased when LAT was shredded compared to ERL indicating greater potential for shredding if grass-clover was harvested at a late developmental stage.

Nutrient composition of microbes flowing in the duodenum was altered by developmental stage, i.e. the microbial concentration of CP and purines were lower and AA tended to be lower when cows were fed LAT compared to ERL. However, no interactions between shredding and developmental stage at harvest were observed for these variables. To our knowledge, such effects of developmental stage has not been shown before. The rumen pH tended to be higher in cows fed LAT compared to ERL, which might be caused by the higher concentration of aNDFom in LAT compared to ERL (Dijkstra et al., 2012). Changes in fermentable matter and pH of rumen fluid can change activity and composition of microorganisms in the rumen and thereby the chemical composition (Weimer et al., 1999), which could be the case in the current experiment.

4.4. CH_4 production and chewing time

The gas production from the cows was investigated since the effects of shredding prior to ensiling and the interaction between shredding and harvest at various developmental stages were expected to cause differences in chewing activity and partly thereby also in the interaction between rumen environment and CH_4 production (Beauchemin, 2018). When grass-clover was shredded, methane production, expressed as L/kg OM digested in the rumen, decreased with 10% (calculated based on Table 8), while the CH_4/CO_2-ratio and RQ tended to be lower and higher, respectively. In addition, when grass-clover was shredded, the ruminal proportion of butyrate increased, the proportion of caproate and concentration of glucose decreased, while the proportion of acetate only tended to decrease. Furthermore, an interaction between shredding and developmental stage was found for the concentration of protein in milk. This showed, despite the lack of effect of shredding on digestibility measures in the rumen, that shredding affected the fermentation pattern in the rumen, which might have had an effect on milk composition. For comparison, Weisbjerg et al. (2018) only observed a lower concentration of NH_3-N and a tendency for higher concentration of L-lactate in rumen fluid, when cows were fed shredded grass-clover silage. Despite ruminal aNDFom digestibility was not improved in the current experiment, cows fed LAT-SHR spent less time for rumination and total chewing time compared to LAT-CON. This indicated a greater potential for shredding for LAT compared to ERL in order to alter the physical structure of forage and favour an increased rate of particle breakdown in the rumen. To support this, the concentration of iNDF in feed is known to be positively correlated to rumination time (Beauchemin, 2018), and although silage iNDF concentration was not affected by shredding in the current experiment, shredding still reduced rumination time for LAT and not for ERL. Shredding has previously shown potential via decreased eating and total chewing time in a normal to relatively mature grass-clover (Weisbjerg et al., 2018).

Cows fed LAT compared to ERL did indeed produce less CH_4, CO_2, and H_2, and rumen fluid pH tended to be higher. The latter was probably due to the lower concentration of total VFA in rumen liquid or could indicate that higher saliva excretion with greater buffering capacity was produced when cows were fed LAT compared to ERL. Higher saliva excretion would correspond to the fact that cows fed LAT compared to ERL spent longer time for rumination and total chewing. The concentration of aNDFom in silages was higher for LAT compared to ERL, which, however, was not accompanied by a higher proportion of acetate in the rumen fluid as previously shown (Sutton et al., 2003), whereas the concentration of NFC in silages was highly correlated with the concentration of total VFA in rumen fluid ($R^2 = 0.92$).

5. Conclusion

The results indicated that shredding increased the silage density and improved the fermentation profile of the silages. Shredding reduced total tract digestibility of aNDFom, but did not affect rumen digestibility of aNDFom in contrast to what was hypothesised. Furthermore, shredding increased the proportion of butyrate in rumen fluid and reduced methane production, expressed as L CH_4 produced per kg OM digested in the rumen. Harvesting grass-clover at a late compared to an early developmental stage decreased the concentration of CP and purines in rumen microbes. In addition, shredding grass-clover harvested at late developmental stage increased the concentration of protein in milk and reduced both rumination and total chewing time compared to a control. However, the lack of interaction between shredding and developmental stage on nutrient digestibility indicated no extra benefits from shredding

grass-clover harvested at late developmental stage.

CRediT authorship contribution statement

Nikolaj Peder Hansen: Methodology, Formal analysis, Investigation, Writing – original draft. **Troels Kristensen:** Conceptualization, Project administration, Writing – original draft. **Marianne Johansen:** Methodology, Formal analysis, Writing – original draft. **Anne Louise Frydendahl Hellwing:** Investigation, Data curation, Writing – original draft. **Peter Waldemar:** Conceptualization, Resources, Funding acquisition. **Martin Riis Weisbjerg:** Conceptualization, Methodology, Validation, Supervision, Writing – original draft.

Conflict of interest

The project was funded by the Green Development and Demonstration Program (GUDP, The Danish Agricultural Agency, The Danish Ministry of Food, Agriculture and Fisheries of Denmark, Copenhagen, Denmark). The project leader was co-author Peter Waldemar, employed at Kverneland Group Kerteminde A/S, who was in charge of developing the machine for shredding. However, we see no conflicts of interest affecting this reporting.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2021.115124.

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5.4 Paper IV

Effect of screw pressing and days of regrowth on grass silage characteristics and quality

Hansen, N.P.,[1] J. Bitsch,[1] S.K. Jensen,[1] M. Ambye-Jensen,[2] M.R. Weisbjerg[1], and M. Johansen[1].

[1]Department of Animal Science, AU Foulum, Aarhus University, 8830 Tjele, Denmark
[2]Department of Engineering, AU Foulum, Aarhus University, 8830 Tjele, Denmark

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Effect of screw pressing and days of regrowth on grass silage characteristics and quality

Hansen N.P.[1], Bitsch J.[1], Jensen S.K.[1], Weisbjerg M.R.[1], Ambye-Jensen M.[2] and Johansen M.[1]
[1]Department of Animal Science, Aarhus University – Foulum, Denmark.; [2]Department of Engineering, Aarhus University, Foulum, Denmark

Abstract

Grass harvested at early and late maturity stages was processed using a twin-screw press to produce a fibrous pulp fraction of which half was ensiled and the other half was pressed a second time before ensiling. For comparison, grass from the same field and maturity stage was mown and wilted before chopping and ensiling. The effects of single and double screw pressing compared with chopped grass on density of the fresh material, fermentation weight loss, and fermentation pattern were investigated in lab-scale silo bags. All combinations of processing and time for regrowth resulted in well-preserved silages with variation in dry matter content, fermentation weight loss, and fermentation products.

Keywords: silage, grass, pulp, density, fermentation loss

Introduction

Biorefinery of fresh grass-clover by screw pressing for production of green protein gives a fibrous pulp as a side stream. The ensiled pulp has potential to increase energy corrected milk yield in dairy cows (Damborg *et al.,* 2019). However, as an effect of screw pressing, the concentration of soluble carbohydrates in the pulp fraction is decreased and may affect the ability of the pulp to ensile sufficiently. Processing the pulp in the screw press a second time is expected to increase the amount of extracted green protein, but also reduces concentration of soluble carbohydrates in the pulp even more. When plant material passes through the screw press, the physical structure of stems and leaves is broken and cell walls are ruptured, and therefore, a better compaction is expected. The objectives of the study were to investigate the effects of processing and days of regrowth of grass on the ability to ensile.

Materials and methods

A grass field (perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*), with 1.4% clover on a dry matter (DM) basis) was harvested late summer 2019 at Aarhus University, Foulum Denmark at early (E) and late (L) maturity stage corresponding to 35 and 44 days of regrowth, respectively. The grass was either mowed and wilted before chopping and ensiling (GS), or harvested and processed immediately using a twin-screw press (5 Mg h^{-1}) yielding a pulp fraction of which half was ensiled (1×P) and the other half was pressed a second time before ensiling (2×P). Each processing was applied within each maturity stage yielding six treatments: GSE, 1×PE, 2×PE, GSL, 1×PL, and 2×PL. Within each treatment, four samples (buckets) where taken, and from each sample, two replicates were made giving eight replicates in total. Each replicate was ensiled in a plastic bag containing (mean ± standard deviation) 580±10.0 g fresh matter. Immediately after sealing and weighing the vacuum bags, silage density was determined as weight of fresh material over the amount of water displaced from each bag (n=4). Fermentation weight loss (FWL) was measured by weighing all bags 0 (n=8), 1 (n=8), 7 (n=8), 14 (n=8), 30 (n=6), and 60 (n=4) days after ensiling. Two replicates per treatment were frozen after weighing on day 14, 30, and 60 after ensiling in order to stop the ensiling process, and these bags were used to investigate the fermentation pattern over time. DM was determined by drying in a forced-air oven at 60 °C for 48 hours. Extracts of silage from each bag were used for pH measurement and analysis of NH_3-N, glucose, L-lactate, and volatile fatty acids. NH_3-N is expressed as a proportion of DM, since total N was only analysed in a

pooled sample from all four buckets at day 0. Statistical analyses were performed in R (version 3.5.2) and the following model was used to analyse data for FWL:

$$Y_{pmdbs} = \mu + \alpha_p + \beta_m + \tau_d + \alpha_p \times \beta_m + \alpha_p \times \tau_d + \beta_m \times \tau_d + \alpha_p \times \beta_m \times \tau_d + A_b + B_{s(b)} + E_{pmdbs}$$

where Y is the dependent response variable, μ is the overall mean, α is the fixed effect of processing (p = GS, 1×P, 2×P), β is the fixed effect of maturity stage (m = E, L), τ is the fixed effect of day after ensiling (d = 1, 7, 14, 30, 60), A is the random effect of bucket within processing × maturity stage (b = 1 to 4), B is the random effect of silage bag within bucket (s = 1, 2), and E is the random residual error assumed to be independent and normal distributed. When analysing data from extracts, B was removed from the model, and τ only had three levels (d = 14, 30, 60). When analysing density, τ, all interactions including τ, A, and B were excluded from the model. FWL was log-transformed and the back-transformed data are shown in the results, without standard error of the mean.

Results and discussion

Processing affected all parameters except pH (Table 1). As expected, density (DM-basis) was highest in 2×P, probably caused by the intensive breakdown of the plants' physical structure due to the mechanical treatment, as shown by Samarasinghe *et al.* (2019). However, in the current experiment, treatment was partly confounded with DM concentration. Relative to total FWL, all silages had a high rate of FWL, especially in the first seven days, and reached a plateau around 30 days after ensiling. Overall, FWL was lowest for 2×P, which also had the highest content of DM. In all silage samples, pH was below 4.3 indicating that all silages were well preserved. GSL had higher concentration of fermentation products than the other silages, probably due to the high water content causing a large production of lactate and acetate. Acetate was present in relatively high concentrations in all silages except 2×P, where also propionate was low, which might reduce aerobic stability (Wilkinson and Davies, 2013). Butyric acid was only detected in three out of the total of 48 samples at concentrations ranging from 2 to 2.8 g kg^{-1} DM, but showed no pattern according to treatments. 2×P had the lowest concentration of NH_3-N, indicating reduced protein degradation during ensiling, as the CP concentration before ensiling was almost identical among type of processing, in agreement with previous studies (Damborg *et al.*, 2019). All silage samples except one had less than 1 g glucose kg^{-1} DM (data not shown). Compared with E, the L-harvested samples had lower DM contents in silage ($P<0.01$, data not shown), lower pH, and higher concentration of L-lactate and propionate, resulting in a smaller FWL.

Conclusions

All treatments resulted in well-preserved silages with variation in DM, FWL, and fermentation products.

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Table 1. Effect of maturity stage and processing (traditional precision chopping, and one or two processes through a screw press) of grass-clover prior to ensiling on initial density, fermentation weight loss (FWL), and fermentation characteristics.[1]

	Day[2]	Treatment[3]						SEM[4]	*P*-value[5]						
		GSE	1×PE	2×PE	GSL	1×PL	2×PL		P	M	D	P×M	P×D	M×D	P×M×D
DM[6], g kg^{-1}	0	289 (13)	286 (13)	401 (21)	207 (3)	324 (18)	401 (17)								
CP[6], g kg^{-1} DM	0	178	181	188	146	157	156								
Density, kg DM (m^3)$^{-1}$	0	278^{b}	293^{b}	370^{a}	189^{c}	316^{b}	391^{a}	11.2	<0.01	0.12		<0.01			
FWL,	1	4.86^{a,D}	5.44^{a,C}	3.13^{b,D}	2.57^{b,C}	3.38^{b,C}	3.12^{b,C}		<0.01	0.03	<0.01	0.39	0.02	<0.01	<0.01
g kg^{-1} FM[7]	7	9.52^{ab,C}	11.0^{a,B}	6.62^{c,C}	10.3^{ab,B}	9.96^{ab,B}	7.51^{bc,B}								
	14	11.2^{ab,B}	12.7^{a,AB}	8.30^{bc,B}	10.5^{ab,B}	10.7^{ab,AB}	7.68^{c,B}								
	30	12.6^{a,AB}	13.9^{a,A}	9.71^{ab,AB}	11.6^{ab,AB}	11.4^{ab,AB}	8.32^{b,AB}								
	60	13.7^{ab,A}	15.3^{a,A}	10.8^{ab,A}	13.0^{ab,A}	12.3^{ab,A}	9.51^{b,A}								
pH	14	4.21	4.18	4.13	4.08	4.11	4.12	0.05	0.57	0.01	0.33	0.12	0.42	0.49	0.05
	30	4.17	4.14	4.24	4.03	4.04	4.10	0.05							
	60	4.26^{a}	4.12^{ab}	4.18^{ab}	4.00^{b}	4.20^{ab}	4.19^{ab}	0.05							
L-Lactate,	14	34.7^{b,B}	35.8^{b}	20.2^{c}	50.7^{a,B}	34.4^{b}	22.8^{c}	2.45	<0.01	<0.01	<0.01	<0.01	0.09	0.87	0.93
g kg^{-1} DM	30	41.5^{b,AB}	38.7^{b}	21.7^{c}	61.6^{a,A}	38.1^{b}	24.0^{c}	2.45							
	60	45.9^{b,A}	39.9^{b}	24.8^{c}	63.0^{a,A}	40.6^{b}	25.9^{c}	2.45							
Acetate,	14	34.2^{bc,B}	34.3^{ab}	22.0^{cd}	41.2^{a}	27.9^{bc,B}	18.5^{d,B}	1.66	<0.01	0.12	<0.01	<0.01	0.79	0.47	0.48
g kg^{-1} DM	30	36.6^{a,AB}	37.4^{a}	27.8^{b}	42.4^{a}	28.6^{b,AB}	21.8^{b,AB}	1.66							
	60	39.6^{ab,A}	36.9^{ab}	26.9^{cd}	44.0^{a}	33.9^{bc,A}	23.8^{d,A}	1.66							
Propionate[8],	14	4.47^{bc}	5.00^{ab}	3.37^{c}	6.42^{a}	4.46^{bc,B}	3.37^{c}	0.423	<0.01	0.01	0.01	<0.01	0.74	0.47	0.50
g kg^{-1} DM	30	4.77^{bc}	5.32^{b}	3.85^{c}	6.96^{a}	4.54^{bc,B}	3.97^{c}	0.388							
	60	4.86^{bc}	5.24^{bc}	4.03^{c}	6.77^{a}	5.60^{ab,A}	4.18^{c}	0.274							
NH_3-N,	14	1.07^{ab,B}	1.31^{a,B}	0.662^{c,B}	1.37^{a,B}	0.901^{bc,B}	0.653^{c,B}	0.080	<0.01	0.82	<0.01	<0.01	0.28	0.60	0.37
g kg^{-1} DM	30	1.42^{b,A}	1.46^{b,AB}	0.927^{c,AB}	1.89^{a,A}	1.14^{bc,B}	0.966^{c,A}	0.080							
	60	1.69^{a,A}	1.74^{a,A}	1.21^{b,A}	1.85^{a,A}	1.56^{ab,A}	1.24^{b,A}	0.080							

[1] Values within same line with different lowercase superscripts differ between treatments ($P<0.05$); Values within same treatment and item with different uppercase superscripts differ over time ($P<0.05$).

[2] Days after ensiling.

[3] Chopped and ensiled at early harvest (GSE), pulp ensiled from early harvest pressed once (1×PE), pulp ensiled from early harvest pressed twice (2×PE), chopped and ensiled at late harvest (GSL), pulp ensiled from late harvest pressed once (1×PL), pulp ensiled from late harvest pressed twice (2×PL).

[4] Highest standard error of mean for LS-mean within row is given.

[5] P = processing, M = maturity stage, D = day of ensiling, P×M = interaction between P and M, P×D = interaction between P and D, M×D = interaction between M and D, P×M×D = interaction between P, M, and D.

[6] Dry matter (DM) and crude protein (CP) in material prior to ensiling. Standard deviation given in brackets for DM (n=8). For CP, n=1.

[7] FM = fresh matter; Back-transformed LSM from log-transformed data.

[8] No detection in three samples (one in GSE 14 days after ensiling and two in GSL 30 and 60 days after ensiling). Samples were not included in the statistical analysis.

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5.5 Paper V

Wet fractionation by screw pressing of grass into pulp and juice can improve fiber digestibility and protein value of pulp for lactating dairy cows

Hansen, N.P.,[1] S.K. Jensen,[1] M. Johansen,[1] A.L.F. Hellwing,[1] M. Ambye-Jensen,[2] M. Larsen,[1] and M.R. Weisbjerg[1].

[1]Department of Animal Science, AU Foulum, Aarhus University, 8830 Tjele, Denmark.
[2]Department of Biological and Chemical Engineering, Aarhus University, 8000 Aarhus C, Denmark.

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Interpretive Summary

Fiber digestibility and protein value of pulp silage for lactating dairy cows – effects of wet fractionation by screw pressing of perennial ryegrass

Hansen et al.

Protein for monogastric nutrition can be extracted from green forages along with water-soluble carbohydrates and minerals during fractionation using a screw press in a biorefinery, producing a protein-rich juice and fiber-rich pulp. The current experiment shows that silage made of pulp of grass can replace traditionally chopped grass silage in diets for dairy cows, and that fiber digestibility and protein value are improved pending on maturity of grass material.

Fiber digestibility and protein value of pulp silage for lactating dairy cows – effects of wet fractionation by screw pressing of perennial ryegrass

N. P. Hansen,[1] S. K. Jensen,[1] M. Johansen,[1] A. L. F. Hellwing,[1] M. Ambye-Jensen,[2] M. Larsen,[1] and M. R. Weisbjerg[1]

[1]Department of Animal Science, AU Foulum, Aarhus University, 8830 Tjele, Denmark

[2]Department of Biological and Chemical Engineering, Aarhus University, 8000 Aarhus C, Denmark

Corresponding author: Nikolaj Peder Hansen, Blichers Allé 20, 8830 Tjele, Denmark. Phone: +45 28605590. E-mail: nikolaj.hansen@anis.au.dk

ABSTRACT

The aim of the study was to investigate the effects of substituting silage of chopped grass with silage of pulp from grass, fractionated once or twice in a screw press-based biorefinery, on fiber kinetics, protein value, and production of CH_4 in dairy cows. Six lactating Holstein cows in mid-lactation, cannulated in the rumen, duodenum, and ileum, were used in an incomplete 6 × 4 Latin square design with a 2 × 3 factorial arrangement of treatments. Perennial ryegrass was harvested in third regrowth from the same field at early and late developmental stage (35 and 44 days of regrowth, respectively) and subjected to one of three types of processing within each developmental stage. Grass was either harvested for normal silage making (mowed, wilted, chopped, and ensiled), or harvested fresh and fractionated using a screw-press. Half of the pulp from the first fractionation was ensiled, whereas the other half of the pulp was rehydrated, fractionated a second time, and pulp hereof was ensiled. The silages were used with concentrates to make total mixed rations (TMR) based on either chopped grass silage (GS), silage of pulp fractionated once (1×P), or silage of pulp fractionated twice (2×P), harvested either at early or late developmental stage resulting in six different TMR treatments (EGS, E1×P, E2×P, LGS, L1×P, L2×P). The TMR were fed for ad libitum intake and samples of intestinal digesta and feces were collected for determination of digestibility. Compared to chopped grass silage, pulp silage from one and two fractionations of grass, respectively, resulted in a linearly increased concentration of crude protein (CP) and neutral detergent fiber (NDF), whereas organic matter digestibility determined in vitro decreased linearly. Substituting GS with 1×P and 2×P, respectively, resulted in a linear total decrease in dry matter intake (DMI) of 2.6 kg/d. Substituting LGS with L1×P and L2×P, respectively, increased ruminal NDF digestibility linearly. However, substitution of GS with 1×P and 2×P, respectively, tended to decrease rate of degradation of digestible NDF and decreased rate of passage of indigestible NDF, while the amount of NDF digested in the rumen increased. An interaction between processing and developmental stage was observed for protein value (g AA digested in the small intestine per kg of

DMI), as the protein value linearly increased with a total of 23%, when substituting EGS with E1×P and E2×P, respectively, whereas processing had no effect on protein value for grass harvested at late developmental stage. The CH_4 yield (L/kg of DMI), but not intensity (L/kg of energy-corrected milk), increased linearly when substituting EGS with E1×P and E2×P, respectively. Quadratic effects were only observed for few of the main variables, such as true CP digestibility in the rumen and rate of passage of indigestible NDF, when substituting GS with 1×P and 2×P, respectively. This study showed that pulp silage of fractionated grass could serve as feed for dairy cows under different feeding regimes, since the fiber digestibility and protein value improved, but further research investigating effects of physical processing of forage on fiber kinetics is required.

Key words: fiber kinetics, forage, methane, perennial ryegrass, ruminant.

INTRODUCTION

In a biorefinery, green forages are fractionated into a fibrous pulp, which can be used in ruminant nutrition, and a protein-rich liquid, suited for meeting the increased global demand for sustainable animal based protein. Green forages such as grass, clover, and lucerne are used for fractionation, since they provide high yields of CP per ha (Wilkins and Jones, 2000) and the CP can be utilized efficiently by monogastrics if it is fractionated from the fibrous part during biorefining (Pirie, 1978). However, on DM basis, extraction of protein from forages still yields a quantitatively dominating side-stream of fibrous pulp, since approximately 65% of DM is recovered in the pulp (Damborg et al., 2020). This pulp might suit as a feedstuff for dairy cows, and thereby improve the sustainable integrity of the concept of biorefining.

Substitution of whole plant silages with pulp of the fractionated whole plant or whole plant silage, has shown that milk production in dairy cows increased (Damborg et al., 2019), tended to decrease (Savonen et al., 2019), and decreased (Sousa et al., 2022). Furthermore, DMI was either

not affected (Damborg et al., 2019; Sousa et al., 2022) or increased at medium inclusion rate (Savonen et al., 2019), when cows were fed silage pulp compared to the corresponding whole plant silage. The effects were probably attributed to increased NDF digestibility and protein value (Damborg et al., 2018; Damborg et al., 2019), which might be further altered by the number of fractionations of the harvested plant material.

During fractionation, the plant is subjected to heavy physical maceration, with the aim of extracting most possible soluble protein, while the plant material is reduced in size, partly defibrillated, and the surface area increased, which potentially could increase digestibility (Buxton and Redfearn, 1997). The DM yield is higher when forage is harvested at late compared to early developmental stage, but NDF digestibility is lower (Rinne et al., 1997) due to increased lignification and stem proportion, which implies a potential for obtaining higher NDF digestibility in pulp of well developed forages compared to whole plant.

The heavy maceration of plant particles during fractionation probably results in different sizes and structures of pulp particles relative to chopped whole plant particles. Consequently, fractional rate of ruminal degradation and passage out of the rumen might have been affected (Buxton and Redfearn, 1997), such that NDF digestibility of the treated plant material, caused by fractionation in the current study, is different compared to that of the whole plant, as seen for other types of physical processing (Koegel et al., 1992; Hansen et al., 2021). Moreover, fractionation of grass-clover have resulted in contradicting effects on OM digestibility (**OMD**) of pulp silage and whole plant silage depending on whether a conventional laboratory method or in vivo experiment was used for determination (Damborg et al., 2019). Therefore, actual knowledge on effects of fractionation on in vivo determined rumen kinetics of pulp compared to the whole plant is needed to relate effects on degradation to milk performance.

The proportion of soluble CP is lower in pulp compared to the whole plant (Damborg et al., 2018) and consequently, the proportion of RUP is higher in pulp, leaving a larger proportion of feed

CP for potential small intestinal digestion. However, in vivo estimation of AA digestion in the small intestine is needed. Hellwing et al. (2018) reported higher CH_4 yield (L/kg of DMI) in heifers fed pure pulp silage compared to pure silage of the whole plant. This is also be expected for dairy cows, as the NDF digestibility probably is higher in pulp compared to the whole plant (Boadi et al., 2004), but dairy cows are often fed concentrate alongside the silages, which might affect the outcome.

The objective of the current study was to investigate the effects of substituting whole plant silage of chopped grass with pulp silage from grass fractionated once or twice at two developmental stages from the same field on nutrient digestion and production of CH_4 in dairy cows. We hypothesize that, compared to whole plant grass silage, increasing the number of fractionations results in pulp silage having 1) increased NDF digestibility, 2) reduced ruminal CP digestibility and increased small intestinal digestion of AA, and 3) increased CH_4 yield.

MATERIALS AND METHODS

The experiment complied with the guidelines set out by the Danish Ministry of Environment and Food (2014) Law No. 474 (May 15, 2014) concerning animal experiments and care of animals used for scientific purposes.

Experimental silages

A field of perennial ryegrass (Lolium perenne L., a mixture of the varieties ‘Garbor’, ‘Bovini 1’, ‘Vsaqui’, and ‘Masai’) was established in 2018 on a location near Tjele, Denmark (56.49° N 9.60° E). The grass was harvested in third regrowth at early (35 days of regrowth) and late (44 days of regrowth) developmental stage in 2019 on August 27 and September 4, respectively. Within each developmental stage, grass was either mowed (7 cm stubble height), wilted, and chopped (JF FCT 900, Kongskilde Industries A/S; 15 mm theoretical length of chopping) before being ensiled or harvested (GT 140, Future Grass technology Ltd.) whole at 7 cm stubble height and brought

directly to the biorefinery demonstration platform in AU Foulum. The grass was fractionated into a solid pulp fraction and a liquid fraction using a twin screw press (P25, CirTech A/S). Half of the pulp produced during fractionation was ensiled in airtight plastic barrels (200 L; Jysk Emballage Rens) without addition of additives. The other half was rehydrated at a ratio of 2:1 (pulp:H_2O) and fractionated a second time in the same screw press before being ensiled in the same way as the pulp from the first fractionation. Within each developmental stage, 10 samples of grass, collected by grab-sampling during delivery of harvested material for the biorefinery, were pooled and divided into stems (leave sheath, stem, and flower) and leaves to determine the stem proportion on DM basis (60°C for 48 h in air-forced oven).

Experimental design, Animals and Housing

Six multiparous Holstein cows cannulated in the rumen (#1C, Bar Diamond Inc.), duodenum, and ileum (simple T-shaped; 25 mm diameter) were used in the current study. The cows were housed in a tie-stall with rubber mattresses, saw-dust as bedding material, and given free access to water. At the beginning of the experiment, the cows averaged (mean ± standard deviation): ECM yield, 30.7 ± 3.7 kg/d; DMI, 20.6 ± 2.6 kg/d; DIM, 176 ± 93 d. The six experimental silages were mixed into six treatments and were fed to the cows that were randomly assigned to the treatments in an incomplete 6 × 4 Latin square design with four periods and a 2 × 3 factorial arrangement of treatments; 2 developmental stages (early and late) and 3 levels of processing (chopped, fractionating once, and fractionating twice). Each experimental period lasted 21 d and constituted a period for adaptation, digesta sampling, and gas exchange measurement. The experimental periods started in a staggered order for the six cows, since the capacity for measuring gas exchange was limited to four cows at a time (described below). Therefore, each reference given to a day within a period in the following sections refers to four cows, and the number given in brackets refers to the last two cows.

Composition of experimental silages are given in Table 1. The silages were fed as a TMR and consisted of 65.00, 6.82, 26.40, 1.36, and 0.46% on DM basis of experimental silage, soybean meal (75 g ash, 91 g NDF and 501 g CP/kg of DM, respectively, 18 g indigestible NDF (**iNDF**)/kg of NDF and 148 g soluble N/kg of N), rolled wheat (15 g ash, 102 g NDF and 114 g CP/kg of DM, respectively, 180 g iNDF/kg of NDF and 264 g soluble N/kg of N), mineral mix (VM2 grøn, Vilofoss; 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 IU/g; vitamin D_3, 190 IU/g; vitamin E, 4000 IU/kg), and mono calcium phosphate, respectively. The six types of TMR were based on silage of either chopped grass (**GS**), pulp from one fractionation (**1×P**), or pulp from two fractionations (**2**×**P**), harvested either at early or late developmental stage. In combination, the six TMR treatments were denoted **EGS**, **E1×P**, **E2×P**, **LGS, L1**×**P**, and **L2**×**P**. The TMR were mixed in a Cormall auger feed-mixer (Cormall A/S) once weekly, vacuum-packed in plastic bags (130 µm thick; 12 kg/bag), and stored in a fridge at maximum 4°C until feeding. The cows were fed for ad libitum intake twice daily at 0715 and 1710 h; feed refusals were removed and weighted before the evening feeding, and the amount of new feed offered were adjusted aiming at 10% refusals. The cows were milked twice daily at 0600 and 1610 h. Two external markers were used to determine the digestibility. The markers were added directly in the rumen in separate degradable filter paper bags (10 g chromium(III) oxide and 13 g titanium(IV) dioxide) during each milking.

Sampling and recording

Samples of silages, soybean meal, and wheat were collected upon mixing of the TMR on d 7 (5) and d 14 (12) within each period and stored at -20°C. After the experiment, thawed samples of silage were pooled within silage type and period (n = 4) and thawed samples of each concentrate were pooled within period 1 and 2 and period 3 and 4 (n = 2). Photos using a microscope with 125 × magnification were taken of each silage to illustrate the visual differences in particle structure

(Figure 1). Extracts of silages were prepared for analysis of fermentation products and buffer capacity by homogenizing 100 g of the pooled samples of silages in 1 L of water. After centrifuging the homogenate, pH was measured in the supernatant, which was stored in duplicate (with and without 25% meta-phosphoric acid) at -20°C until analysis. Pooled samples of silages and concentrates were stored at -20°C until chemical and in situ analysis.

Samples of TMR and TMR refusals were collected during five consecutive days starting on d 12 (10) and d 13 (11), respectively, to determine DM concentration (60°C for 48 h), and subsequently to calculate the average DMI per cow per period. Milk yield and composition was determined on the same days as DMI and also averaged per cow per period.

To cover the diurnal variation, twelve samples of duodenal digesta (400 mL), ileal digesta (200 mL), and feces (300 mL) were collected during d 13 (11) to 17 (15) in each period (d 13 (11): 1000 h, 1800 h; d 14 (12): 0200 h, 1200 h, 2000 h; d 15 (13): 0400 h, 1400 h, 2200 h; d 16 (14): 0600 h, 1600 h, 2400 h; d 17 (15): 0800 h). The samples were pooled and stored at -20°C before chemical analysis. On the same 12 time points, rumen fluid was collected from the ventral rumen sac through the rumen cannula, using a plastic syringe mounted to a suction strainer. Immediately after sampling, rumen fluid pH was measured and the samples were stored at -20°C before chemical analysis.

Gas exchange was measured for each cow during a 72-h period from d 17 to 20 (18 to 21) in individual 17 m^3 respiration chambers (Hellwing et al., 2012). Since only four chambers were available, the cows were staggered in a way that, within each period, four cows entered the chambers first and then the last two cows. During the period of gas exchange measurement, chambers were accessed twice daily in order to milk and feed the cows, and clean the stalls. Samples of TMR and TMR residues were collected on the three days during the gas measurement to determine DM concentration (60°C for 48 h) and subsequently DMI in the period of gas exchange measurement for each cow per period. Milk yield was determined for the same three days and also

198 averaged per cow per period for the period of gas exchange measurement. The concentrations of
199 CH_4, CO_2, O_2, and H_2 were measured every 12.5 min in all periods in air exhaust from each of the
200 chambers and the background air (inlet air). The air flow was measured using a HFM-200 flow
201 meter with a laminar flow element (Teledyne Hastings Instruments). The concentration of CH_4 was
202 measured using an infrared sensor (VIA-510, Horiba instruments), CO_2 using an infrared sensor and
203 O_2 with a paramagnetic sensor, both from Columbus Instruments International, and the
204 concentration of H_2 using an electrochemical sensor (3HYT CiTiceL, Honeywell International Inc.).
205 Gas exchange data was deleted from time points, where chamber doors were open. The 24 h gas
206 exchange was calculated as accumulated gas over the total measuring period divided by the total
207 measuring time in min multiplied with 1440 min. Recovery tests were performed before the
208 experiment, between periods, and after the experiment for individual chamber correction. In total,
209 57 CO_2 recovery tests with a recovery of 99.3 ± 1.4% and 25 CH_4 recovery tests with a recovery of
210 99.1 ± 1.6% were performed. For O_2 and H_2, an average of the recovery of CO_2 and CH_4 was used.
211 Gas production is reported in L under standard conditions (0°C, 101.325 kPa).
212 To avoid carry over-effects on measurements of gas exchange, rumen evacuations were
213 performed at 1145 h on d 15 for the two cows entering the respiration chambers last, and at 1145 h
214 on d 21 for the four cows entering the respiration chambers first. The time point for rumen
215 evacuation was chosen to obtain samples and recordings representative for the diurnal variation in
216 rumen pools (Lund, 2002). All rumen content was placed manually into a sieve basket, through
217 which the liquid fraction of rumen content was allowed to run into a tub, while the solid fraction
218 remained in the sieve basket. After weighing and mixing, subsamples were taken from the solid and
219 liquid fractions, and three composited samples (500 g) were made proportionally to the weight of
220 each fraction, making representative samples for the whole rumen content. The composited samples
221 were either used for DM determination (60°C for 48 h) or stored at -20°C until chemical and in situ
222 analysis. At 1000 h on d 17 (15), 3.0 L of rumen fluid was collected for harvest of microbes by

223 differential centrifugation as described by Johansen et al. (2017). Milk samples were collected at
224 two occasions; during six consecutive milkings starting on d 13 (11) and during the last four
225 consecutive milkings, in the period where gas exchange was measured.
226
227 ***Chemical analyses***
228 Pooled samples of silages, concentrates, purified microbes, duodenal digesta, ileal digesta,
229 feces, and rumen contents were freeze-dried and milled through a 1 mm screen (ZM 200 mill,
230 Retsch GmbH) prior to chemical analysis. In all samples, ash was determined by combustion at
231 525°C for 6 h. Nitrogen was analyzed following the Dumas principle (Hansen, 1989) using a Vario
232 Max CN (Elementar Analysesysteme GmbH) and CP was calculated as N × 6.25. Soluble N was
233 determined in silages and concentrates by extraction in a 39°C borate-phosphate buffer at pH 6.75
234 for 1 h (Åkerlind et al., 2011). All samples, except purified microbes, were analyzed sequentially
235 for NDF, ADF, and ADL following the ANKOM procedure using heat-stable α-amylase and
236 sodium sulphite (Mertens, 2002) and corrected for residual ash after the ADL procedure. The
237 concentration of hemicellulose was calculated as the difference between NDF and ADF, and
238 cellulose was calculated as the difference between ADF and ADL. To determine digesta flow,
239 duodenal digesta, ileal digesta, and feces were analyzed for chromium(III) oxide by
240 spectrophotometry after oxidation with sodium peroxide chromate (Schürch et al., 1950), and for
241 titanium(IV) dioxide as described by Myers et al. (2004) with the modification that 15 instead of 10
242 mL of 30% hydrogen peroxide were added and that five additional drops of hydrogen peroxide
243 were added before measuring the absorbance. To determine microbial synthesis in the rumen,
244 purified microbes and duodenal digesta were analyzed for purines by spectrophotometry as
245 described by Zinn and Owens (1986) and modified by Thode (1999). The AA were analyzed in
246 silages, concentrates, purified microbes, duodenal digesta, and ileal digesta using UPLC (Dahl-
247 Lassen et al., 2018), and the total amount of AA was calculated as the sum of Ala, Arg, Asp, Cys,

Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, and Val. The silage in vitro OM digestibility was determined following the procedure by Tilley and Terry (1963), where samples of silage were incubated in rumen fluid for 48 h and subsequently in a HCl and pepsin solution for 48 h. The in vivo OMD was then calculated as 4.10 + 0.959 × in vitro OM digestibility as described in Åkerlind et al. (2011).

Indigestible NDF (**iNDF**) was determined in silages, concentrates, rumen contents, and feces by incubating Dacron bags (12 µm pore size, Saatitech S.p.A.) with 2 g freeze-dried and milled samples (1.5 mm; Pulverisette 15, Fritsch GmbH) in three non-lactating cows (3 replicates; one bag per cow per pooled sample) for 288 h as described by Krämer et al. (2012). Residues remaining in bags after incubation were quantitatively transferred to filter crucibles and analyzed for NDF using the Fibertech^{TM} M6 system (Foss Analytical) and referred to as iNDF.

In samples of rumen fluid and silage extracts containing meta-phosphoric acid, individual VFA was analyzed using GC (Kristensen et al., 1996), ammonia N was analyzed using a Cobas Mira autoanalyzer (Triolab A/S), and L-lactate and glucose were analyzed using an YSI 2900D (YSI Inc.) following the immobilized oxidase electrode technique (Mason, 1983). The sum of individual VFA and L-lactate was referred to as short-chained FA (**SCFA**). Buffer capacity was measured on silage extracts without meta-phosphoric acid as the meq alkaline required to raise pH from 4 to 6 pr. 100 g DM (Playne and McDonald, 1966) using a Titrator Excellence T7 (Mettler Toledo).

Calculations

The DM flow in duodenum and ileum and the DM output in feces were calculated for each marker, averaged, and then used for calculating nutrient flow and output. Assuming that purines in duodenal digesta was only of microbial origin, duodenal flow of microbial DM (kg/d) was calculated as: duodenal flow of purines (kg/d) / purine concentration in purified microbes (g/kg DM) × 1000. Using duodenal flow of NDF have previously yielded unreliable ruminal digestibility

of NDF (Brask et al., 2013), and therefore, ileal flow of NDF was used instead. The true ruminal digestibility of DM, OM, and CP was calculated by correcting for duodenal flow of microbial DM, OM, and CP, respectively. Efficiency of the microbial CP synthesis was expressed as the duodenal flow of microbial CP per kg of NDF or OM truly degraded in the rumen. The rumen fractional rate of degradation (**k_d**; %/h) of digestible NDF (**DNDF**; calculated as NDF minus iNDF) was calculated as: ([DNDF intake (kg/d) – fecal output of DNDF (kg/d)] / 24) / Rumen pool of DNDF (kg) × 100. The rumen fractional rate of passage (**k_p**; %/h) of iNDF was calculated as: [Fecal flow of iNDF (kg/d) / 24] / Rumen pool of iNDF (kg) × 100.

Yield weighted averages of fat, protein, and lactose were used to calculate the ECM (3.14 MJ/kg) yield (Sjaunja et al., 1990) across days within the digesta sampling period and within the period of gas exchange measurement, where milk yield was measured: ECM yield = milk yield (kg) × [(38.3 × fat (g/kg) + 24.2 × protein (g/kg) + 15.71 × lactose (g/kg) + 20.7) / 3,140], where lactose is lactose monohydrate. The N use efficiency was calculated as: [milk protein (kg/d) / 6.38] / N intake (kg/d) × 100. The CH_4 production was expressed in relation to the DMI and ECM yield determined during the period of gas exchange measurement, and referred to as CH_4 yield and CH_4 intensity, respectively.

Statistical Analysis

The statistical analyses were conducted using R 4.0.4 (R Core team, 2021) and the effects of developmental stage and processing were analyzed using the mixed linear model in Equation 1 with the '*lmer*' function from the '*lme4*' package (Bates et al., 2015).

$$Y_{dptc} = \mu + \alpha_t + \tau_p + A_c + \varepsilon_{dptc} \quad (1)$$

For the model in Equation 1, Y_{dptc} is the dependent response variable (n = 4), μ is the overall mean, α_t is the fixed effect of treatment (t = EGS, E1×P, E2×P, LGS, L1×P, L2×P), τ_p is the fixed effect of period (p = 1, …, 4), and A_c is the random effect of cow (c = 1, …, 6). The random effect

298 of cow A_c and the residual error ε_{dptc} were assumed to be normal distributed with zero mean and
299 variance σ_A^2 and σ_ε^2, respectively.

300 When analyzing the chemical composition of silages (Table 1), the model in Equation 1 was
301 also used, except that the random effect of cow A_c was excluded. Variables for rumen fluid pH, the
302 concentrations of ammonia N and glucose in rumen fluid, and rumen fluid SCFA proportions were
303 analyzed using the model in Equation 1 and including the fixed effect of sampling time, the 2-way
304 interaction of treatment and sampling time, and sampling time within cow and period as repeated
305 measurement. However, the 2-way interaction was not significant for any of the response variables.
306 The response variables were therefore averaged per cow per period and tested using the model in
307 Equation 1. The experimental design resulted in four observations per treatment unless otherwise
308 stated in the tables. However, data on digesta from ileum from one cow receiving EGS, E1×P,
309 E2×P, and LGS were not available due to cannula problems.

310 The '*emmeans*' package was used to obtain least square means and highest standard error of
311 means (**SEM**), which are given in the tables. The '*glht*' function from the '*multcomp*' package
312 (Hothorn et al., 2021) was used to extract *P*-values for contrasts of testing the effect of
313 developmental stage (early vs. late; given in tables as Dev), *P*-values for the linear and quadratic
314 effect of substituting GS with 1×P and 2×P, respectively (given in tables as L and Q for linear and
315 quadratic test, respectively), and *P*-values for the interaction between developmental stage and the
316 linear and quadratic effect of substituting GS with 1×P and 2×P, respectively (given in tables as L ×
317 Dev and Q × Dev, respectively). The *P*-values for contrasts of testing the linear and quadratic effect
318 of substituting GS with 1×P and 2×P, respectively, within each developmental stage, were also
319 extracted if L × Dev or Q × Dev were significant; however, these are only reported in the text when
320 relevant. The linear and quadratic effects were assessed to describe the expected trend in each
321 response variable, when substituting chopped grass silage with pulp silage from grass fractionated

once and twice, respectively. Statistical significance was regarded when $P \leq 0.05$ and as tendencies when $0.05 < P \leq 0.10$.

RESULTS

Silages and Composition of TMR

The stem proportion averaged 7.5 and 11.5% on DM basis (not shown) for grass harvested at the early and late developmental stage, respectively. The NDF and CP concentrations were higher (591 vs. 549 g NDF/kg of DM; $P < 0.01$) and lower (162 vs. 186 g CP/kg of DM; $P < 0.01$), respectively, and iNDF proportion higher (103 vs. 93 g iNDF/kg of NDF; $P < 0.01$) in silages of grass harvested at late compared to early developmental stage (Table 1). Moreover, silage of grass harvested at late compared to early developmental stage had lower (74 vs. 77%; $P < 0.01$) in vitro determined OMD.

Compared to chopped grass silage, pulp silage from one and two fractionations of grass, respectively, harvested only at early developmental stage, resulted in a quadratic response ($P < 0.01$) for DM concentration, and when grass harvested at late developmental stage was processed, DM concentration increased ($P < 0.01$) linearly. Compared to chopped grass silage, pulp silage from one and two fractionations of grass, respectively, had linearly decreased ($P < 0.01$) ash concentration and linearly increased ($P < 0.01$ and $P = 0.02$, respectively) concentrations of NDF and CP, but a significant interaction ($P = 0.03$) showed that the change for ash concentration was greater, when comparing processing for grass harvested at early compared to late developmental stage. A quadratic effect ($P = 0.02$) showed that the reduction found in the proportion of soluble N between chopped grass silage and pulp silage from the first fractionation, was smaller than the reduction found between pulp silage from the first fractionation and pulp silage from the second fractionation. Moreover, an interaction ($P < 0.01$) showed that the total decrease in soluble N proportion was higher, when processing grass harvested at early (-288 g N/kg of total N; $P < 0.01$)

compared to late (-229 g N/kg of total N; $P < 0.01$) developmental stage. An interaction ($P = 0.03$) showed that, compared to chopped grass silage, pulp from one and two fractionations, respectively, had a linear total reduction of in vitro determined OMD that was higher, when processing grass harvested at early (-3.5%-units, $P < 0.01$) compared to late (-1.9%-units; $P < 0.01$) developmental stage. Figure 1 shows the visual effects on structure of forage material of processing of grass harvested at early and late developmental stage.

An interaction ($P < 0.01$) showed that the total decrease in silage pH and buffer capacity and the total increase in concentration of L-lactate was higher, when processing grass harvested at late compared to early developmental stage.

Feed Intake, Duodenal Flow, and Weight Changes

Substitution of GS with 1×P and 2×P, respectively, linearly decreased ($P < 0.01$ for both; Table 2) DMI and CP intake with a total of 2.6 and 0.4 kg/d, respectively, whereas NDF intake linearly increased ($P < 0.01$) with a total of 1.2 kg/d. In table 3, an interaction ($P < 0.01$) showed that substitution of EGS with E1×P and E2×P, respectively, linearly increased (25 g/kg of DMI; $P < 0.01$) duodenal flow of AA of feed and endogenous origin more compared to substitution of LGS with L1×P and L2×P, respectively (8.6 g/kg of DMI; $P < 0.01$). Substitution of GS with 1×P and 2×P, respectively, linearly decreased ($P = 0.02$) duodenal flow of microbial AA; however, an interaction tended ($P = 0.08$) to show that only substitution of LGS with L1×P and L2×P, respectively, decreased ($P < 0.01$) duodenal flow of microbial AA, and not when substituting EGS with E1×P and E2×P, respectively ($P = 0.74$). Substitution of GS with 1×P and 2×P, respectively, had no effect on daily BW change, and from the beginning to the end of the experiment, total BW change averaged -6.8 kg across treatments.

Digestibility, Rumen Kinetics, and Rumen Fluid

372 Substitution of LGS with L1×P and L2×P, respectively, resulted in a linear total increase ($P <$
373 0.01) of 10%-unit on ruminal NDF digestibility, whereas substitution of EGS with E1×P and E2×P,
374 respectively, had no effect ($P = 0.91$) on ruminal NDF digestibility (Table 3). An interaction ($P <$
375 0.01) showed that the linear total reduction in true CP digestibility in the rumen was higher (-16%-
376 units and $P < 0.01$ vs. -5.1%-units and $P = 0.01$, respectively), when substituting EGS with E1×P
377 and E2×P, respectively, compared to LGS with L1×P and L2×P, respectively. But the response was
378 also quadratic ($P < 0.01$), showing that the decrease was higher when substituting 1×P with 2×P
379 compared to substituting GS with 1×P. Small intestinal digestibility of AA linearly increased ($P =$
380 0.05) by a total of 1.9%-units, when substituting GS with 1×P and 2×P, respectively. An interaction
381 ($P = 0.03$) showed that the amount of AA digested in the small intestine increased linearly (22 g/kg
382 of DMI; $P < 0.01$) only when EGS was substituted with E1×P and E2×P, respectively (Table 4). An
383 interaction ($P = 0.01$) showed that substituting LGS with L1×P and L2×P, respectively, resulted in a
384 quadratic response ($P = 0.01$) for the efficiency of the microbial CP synthesis (g CP/kg of NDF
385 digested in the rumen) such that the reduction in efficiency was higher, when substituting LGS with
386 L1×P compared to substituting L1×P and L2×P. However, across developmental stage, substitution
387 of GS with 1×P and 2×P, respectively, decreased ($P < 0.01$) the efficiency of the microbial CP
388 synthesis with a total of 131 g microbial CP/kg of NDF digested in the rumen. The efficiency of the
389 microbial CP synthesis was not affected by processing, when related to OM digested in the rumen.
390 The total content of material (fresh matter and DM) in the rumen as well as the rumen pool size
391 of each nutrient (OM, NDF, iNDF, and DNDF) increased linearly ($P < 0.01$ for all), when
392 substituting GS with 1×P and 2×P, respectively (Table 5). Substitution of GS with 1×P and 2×P,
393 respectively, tended to linearly reduce ($P = 0.06$ and $P = 0.05$, respectively) k_d of DNDF from 5.10
394 to 4.44 %/h, and k_p of iNDF from 1.66 to 1.44 %/h. However, an interaction ($P = 0.03$) showed that
395 only substitution of EGS with E1×P and E2×P, respectively, resulted in a quadratic response ($P =$

0.04), such that k_p of iNDF decreased when substituting EGS with E1×P and increased when substituting E1×P with E2×P.

Rumen pH and concentration of total SCFA in rumen fluid were not affected by treatments (Table 6). Substitution of GS with 1×P and 2×P, respectively, increased ($P < 0.01$ and P = 0.01, respectively) the proportion of acetate and the acetate:propionate ratio linearly, whereas the proportion of butyrate decreased linearly.

Milk and production of CH_4

The ECM yield averaged 23.9 kg/d across treatments, and was not affected by substitution of GS with 1×P and 2×P, respectively (Table 7). The feed efficiency (kg of ECM/kg of DMI) linearly increased ($P < 0.01$) when substituting GS with 1×P and 2×P, respectively, and an interaction tended ($P = 0.09$) to show that the increase in feed efficiency only occurred, when substituting EGS with E1×P and E2×P, respectively ($P < 0.01$). Substitution of GS with 1×P and 2×P, respectively, linearly decreased ($P = 0.01$) the CH_4 production by a total of 49 L/d, whereas an interaction ($P = 0.01$) showed that only substitution of EGS with E1×P and E2×P, respectively, linearly increased ($P < 0.01$) CH_4 yield (L/kg of DMI) by a total of 8.7% (Table 8). CH_4 intensity (L/kg of ECM) was not affected, whereas the H_2 production (L/d) linearly decreased ($P < 0.01$) when substituting GS with 1×P and 2×P, respectively.

DISCUSSION

This study compared traditionally chopped grass silage with pulp silage from a biorefinery on fiber digestibility, feed protein value, and production of CH_4 using lactating dairy cows. The grass for traditional ensiling was cut and the grass fractionated in the biorefinery was harvested on the same day in the same field. It is one of few studies, which has investigated the value of pulp silage from green forages in vivo for lactating dairy cows, and it is the first in vivo study to enlighten the

passage kinetics and digestion of fiber in the rumen and to quantify the protein value and the production of CH_4 from dairy cows fed pulp silage. Overall, we found that the processing interacted with the developmental stage of the grass at harvest. Fiber digestibility improved, when substituting chopped grass silage with pulp silage of grass harvested at late developmental stage. In contrast, the protein value (g AA digested in the small intestine per kg of DMI) improved and CH_4 yield increased, when substituting chopped grass silage with pulp silage of grass harvested at early developmental stage.

Silages

All silages were well preserved and appeared of good quality upon usage. The chemical composition of silages reflected the prolonged regrowth since grass harvested at late compared to early developmental stage had higher NDF and CP concentration, higher iNDF proportion, and lower in vitro determined OMD.

Silages of chopped grass obtained DM concentrations lower than the 320 g/kg aimed for. This was because grass for the chopped grass silage, harvested at early and late developmental stage was wilted for only 24 and 16 h, respectively, to avoid loss of nutrients due to forecasted rain. Grass harvested for fractionation had an initial DM concentration of ~170 g/kg, and despite water was added to the pulp prior to the second fractionation, the DM concentration continued to increase compared to the first fractionation. The increased DM concentration of pulp silage for each fractionation step was probably driven by intensified disintegration of plant fibers during each fractionation, aiding the extraction of water. The linear increase in CP concentration, when comparing chopped grass silage to pulp silage from the first and the second fractionation, respectively, was probably caused by the loss of additionally and soluble compounds, indicated by e.g. the more than 50% concomitant reduction in ash concentration. At comparable CP concentration as to our study, Damborg et al. (2018) showed no difference in CP concentration

between the whole plant material and the pulp after fractionation, and did also show that a considerably large part of CP remaining in pulp was associated with the fibers (NDF). The latter suggested that complete fractionation of CP from the grass was impossible when using a screw press as in the current experiment. Savonen et al. (2019) and Sousa et al. (2022) reported considerably lower CP concentrations in pulp from silage compared to the whole plant silage. This indicates that fractionation of fresh (as in our study) vs. ensiled forages seems to affect the expected difference in CP concentration between the remaining pulp and the whole plant material. The NDF concentration, when comparing chopped grass silage to pulp silage from the first and the second fractionation, respectively, increased linearly since fiber particles were retained in the screw press during fractionation, and by each fractionation, an additional amount of soluble nutrients were extracted into the liquid fraction. The linearly increased NDF concentration was probably the main factor contributing to the linearly decreased OMD determined in vitro, when comparing chopped grass silage to pulp silage from the first and the second fractionation, respectively.

The loss of easily fermentable carbohydrates during fractionation could have resulted in restrictive fermentation of pulp, but pH was sufficiently low for all silages, and decreased, when comparing chopped grass silage to pulp silage from the first and the second fractionation, respectively. Reduced concentrations of L-lactate and acetate in silages indicated that less acid was required to lower pH and stop fermentation, when comparing chopped grass silage to pulp silage from the first and the second fractionation, respectively. However, the achieved levels of pH and acid could be related to the increased DM concentration (McDonald et al., 1991) and the decreased buffer capacity (McDonald and Henderson, 1962), when comparing chopped grass silage to pulp silage from the first and the second fractionation, respectively. The buffer capacity decreased because of decreased concentrations of ash (i.e. minerals) and soluble N.

NDF digestibility and rumen kinetics

Substituting LGS with L1×P and L2×P, respectively, increased the ruminal digestibility of NDF and DNDF linearly. Ruminal digestibility of NDF depends on the competitive processes of feed particles being either degraded or passing out of the rumen. Using rumen evacuation data, the total decrease of k_d of DNDF numerically reached 0.68%-units when substituting GS with 1×P and 2×P, respectively, and it was unexpected that k_d of DNDF did not increase. For comparison, Damborg et al. (2019) found that the in situ determined k_d of potentially degradable NDF was 0.84%-units higher in pulp silage compared to the whole plant silage. In contrast to what we expected, k_d of DNDF probably decreased, when substituting GS with 1×P and 2×P, respectively, because of the parallel increase (38%) in the rumen pool size of DNDF. The parallel increased pool size of DNDF would dilute any possible increase in degradation of DNDF. Furthermore, our estimation of k_d of DNDF was based on rumen evacuation data obtained at a single time point. Diurnal variations of the rumen pool size of DM and nutrients are known to occur (Huhtanen et al., 2007), which, as addressed by Lund (2002), could potentially interact with our dietary treatments. However, a sensitivity analysis of the current data (not shown) comprising adjustment of the rumen pool size demonstrated that a possible treatment related diurnal variation in rumen pool size resulting in an overestimated pool size of cows fed 2×P compared to GS was far from the only explanation for the decreased k_d of DNDF. To obtain equal k_d of DNDF for GS and 2×P, rumen pool size of DNDF for E2×P and L2×P should have been 22 and 9% lower than observed, respectively, which we judge as being considerably more than expected treatment related bias in average DNDF pool size.

Although daily fecal excretion of iNDF increased with a total of 14% (results not shown), the k_p for iNDF decreased when substituting GS with 1×P and 2×P, respectively, because the rumen pool of iNDF increased even more (34%). In addition, the fractionation of the grass may have caused greater entanglement of pulp silage fibers and particles in the rumen content. Despite lacking data to support these considerations, it is noteworthy that rumen content from cows fed 2×P

clearly seemed more densely packed with a paste-like appearance and less stratification of feed particles, which might have affected fiber passage and degradation kinetics compared to cows fed GS. Despite the interaction between developmental stage and the linear effect of processing was not significant, numerically, iNDF and DNDF pool sizes increased with a total of 47 and 38%, respectively, when substituting EGS with E1×P and E2×P, respectively, while the iNDF and DNDF pool sizes increased with a total of 21 and 37%, respectively, when substituting LGS with L1×P and L2×P, respectively. This could indicate that the decrease in the DNDF:iNDF ratio of rumen content was greater, when grass harvested at early compared to late developmental stage was used to compare pulp silage with chopped grass silage (Lund et al., 2006). A significant interaction showed that the response in the rumen pool size of iNDF was quadratic only when substituting EGS with E1×P and E2×P, respectively, but the reason for this was unknown.

Total tract digestibility of NDF increased with a total of 6.8%-units, when substituting LGS with L1×P and L2×P, respectively, whereas substitution of EGS with E1×P and E2×P, respectively, had no effect on NDF digestibility. Increased digestibility of hemicellulose seemed to be the main driver for this increased NDF digestibility. Likewise, Damborg et al. (2019) observed a 9.5%-units greater total tract digestibility of NDF, when comparing pulp silage to the corresponding chopped whole plant grass-clover silage. However, Damborg et al. (2019) used a forage, where clover constituted 55% on DM-basis of the experimental silage compared to the only-grass forage in our experiment. The maceration of plant fibers during fractionation might affect legume fibers differently from that of grass fibers, since lignin deposition occurs in the xylem of the vascular tissue and only in stems in legumes, whereas lignin is present in almost all tissues and organs in ryegrass (Buxton and Redfearn, 1997). For comparison, total tract digestibility of NDF in the studies by Savonen et al. (2019) and Sousa et al. (2022) has shown not to be different between pulp of silage and the whole plant silage, although silage and not fresh forage was used for fractionation. For application on farms, use of pulp as feed for ruminants includes estimation of fiber digestibility

for feed ration planning. These results suggested that our understanding of the mechanisms of fiber digestion is not unequivocal when comparing pulp to the whole plant. Thus, further research is needed for quantifying the effect of fractionation on the physical properties of the feed particles of pulp and the subsequent variation in effect on fiber digestibility.

When comparing pulp silage to the whole plant silage, Damborg et al. (2019) observed contradicting effects on OMD depending on the technique used for determining digestibility; hence, in vitro showed lower OMD of pulp silage, whereas in vivo showed higher OM digestibility of pulp silage compared to the whole plant silage. In our study, compared to chopped grass silage, one and two fractionations of grass, respectively, also decreased the in vitro determined OMD. However, compared to chopped grass silage, one and two fractionations of grass, respectively, also decreased the apparent total tract digestibility of OM, but only when grass harvested at early and not late developmental stage was processed. Some mismatch between estimates obtained from in vitro and in vivo techniques therefore still seem to occur, when comparing effects of physical processing (i.e. fractionation using a screw press in our experiment) on digestibility. One potential explanation could be the already mentioned masking effect of laboratory processing (drying and grinding) of fractionation effects on digestibility (Damborg et al., 2019).

The concentration of NDF in feed has a greater filling effect than other nutrients, and is therefore negatively correlated to voluntary DMI (Allen, 1996). However, the rumen pool size of DM, NDF, and iNDF increased when substituting GS with 1×P and 2×P, respectively, which indicated that voluntary DMI was not only physically regulated, or that physical regulation changed when feeding pulp silage compared to chopped grass silage, or that physical fill depend on more than NDF. The same increase in rumen pool of NDF and decrease in DMI was observed, when grass silages of increasing maturity stages were fed (Rinne et al., 2002). Based only on the fact that NDF concentration of diets increased with a total of 14%-unit when substituting GS with 1×P and 2×P, respectively, DMI was expected to decrease more due to higher fill than what was observed.

546 However, as indicated by Damborg et al. (2019), where similar DMI but higher NDF digestibility
547 were reported when comparing pulp silage to the whole plant silage, factors including e.g. particle
548 size and particle fragility might also have affected the fill of pulp differently than chopped grass
549 (Allen, 1996). The photos of the six silages (Figure 1) indicated that fractionation affected those
550 factors, which subsequently might have had limited the reduction in the fill value and resulted in the
551 limited decrease in DMI, when substituting GS with 1×P and 2×P, respectively.
552
553 ***Protein digestibility and metabolizable protein***
554 The amount of AA digested in the small intestine per kg of DMI increased only when
555 substituting EGS with E1×P and E2×P, respectively, but not when substituting LGS with L1×P and
556 L2×P, respectively. The AA digested in the small intestine originate from feed AA not digested in
557 the rumen, microbial AA, and endogenous AA. Substitution of GS with 1×P and 2×P, respectively,
558 reduced true CP digestibility in the rumen, which was attributed to the concomitantly reduced
559 proportion of soluble N in silages. These in vivo data were in alignment with previous in situ data
560 (Damborg et al., 2018), where effective CP degradation in the rumen was also lower in pulp
561 compared to the whole plant, but not to the same extent as in our in vivo study. The greater
562 reduction in true ruminal CP digestibility, when substituting EGS with E1×P and E2×P,
563 respectively, compared to substituting LGS with L1×P and L2×P, respectively, suggested that at
564 late compared to early developmental stage, CP was embedded in cell walls that had been more
565 lignified (Keller, 1993). Cellulose and iNDF concentrations were higher in grass harvested at late
566 compared to early developmental stage. Despite the true CP digestibility in the rumen decreased
567 linearly, when substituting GS with 1×P and 2×P, respectively, the response in ammonia N
568 concentration in rumen fluid was quadratic. The quadratic effect indicated that cows fed 1×P
569 generally had higher concentrations of ammonia N in the rumen fluid. The reason for this was
570 unknown, but all estimates were within biological range of ammonia N concentration in the rumen

571 (Abdoun et al., 2006). Consequently, the reduced true ruminal CP digestibility increased the
572 duodenal flow of feed AA (assuming endogenous flow is constant across treatments), when GS was
573 substituted with 1×P and 2×P, respectively. However, the increase was greater, when substituting
574 EGS with E1×P and E2×P, respectively, compared to substituting LGS with L1×P and L2×P,
575 respectively. Substitution of GS with 1×P and 2×P, respectively, reduced the duodenal flow of
576 microbial AA, and the flow tended to be reduced only when substituting LGS with L1×P and L2×P,
577 respectively. This was probably caused by the increased NDF concentration, i.e. slowly digested
578 carbohydrates, when substituting GS with 1×P and 2×P, respectively. Substitution of GS with 1×P
579 and 2×P, respectively, also reduced the efficiency of the microbial CP synthesis, when related to
580 NDF but not OM digested in the rumen. The quadratic response suggested that the efficiency (when
581 related to NDF digested in the rumen) was reduced drastically already when LGS was substituted
582 by L1×P, whereas substitution of EGS with E1×P had minor effects on the efficiency of the
583 microbial protein synthesis.
584 All combined, the increased duodenal flow of feed AA, equal duodenal flow of microbial AA,
585 and increased small intestinal digestibility of AA resulted in improved protein value (g AA digested
586 in the small intestine per kg DMI), when EGS was substituted with E1×P and E2×P, respectively.
587 This was in agreement with Damborg et al. (2018), who also showed higher in situ determined
588 protein value of pulp from fractionated ryegrass, white clover, red clover, and lucerne compared to
589 the whole plant of each forage. However, since the duodenal flow of microbial AA tended to
590 decrease, no improvement of the protein value was obtained, when LGS was substituted with L1×P
591 and L2×P, respectively. The total amount of AA digested in the small intestine (g/d) was not
592 affected since DMI was reduced, when GS was substituted with 1×P and 2×P, respectively.
593 We used medium yielding cows in mid-lactation and all cows had a sufficient supply of MP.
594 Therefore, no further improvements of the milk production was expected from substituting EGS
595 with E1×P and E2×P, respectively, despite it had a greater protein value, i.e. higher MP (Huhtanen

596 and Hristov, 2009). Feed conversion ratio (kg ECM/kg DMI) increased when substituting GS with
597 1×P and 2×P, respectively, and the increase tended to be greatest when substituting EGS with E1×P
598 and E2×P, respectively, compared to substituting LGS with L1×P and L2×P, respectively. The
599 reason was mainly that the ECM yield decreased numerically less, when substituting EGS with
600 E1×P and E2×P, respectively, compared to substituting LGS with L1×P and L2×P, respectively.
601 However, experimental periods lasted only for 21 d and results should therefore be interpreted with
602 care.
603
604 ***Production of CH_4***
605 We expected CH_4 yield (L/kg of DMI) to increase due to the greater amount of NDF digested
606 in the rumen, when substituting GS with 1×P and 2×P, respectively. Fermentation of NDF in the
607 rumen yields primarily acetate with H_2 surplus, and H_2 is used for by methanogens forming CH_4
608 (Boadi et al., 2004). Indeed, CH_4 yield did increase; however, only when substituting EGS with
609 E1×P and E2×P, respectively. Interestingly however, although the amount of NDF degraded in the
610 rumen and the proportion of acetate in rumen fluid increased, the daily production of H_2 decreased
611 when substituting GS with 1×P and 2×P, respectively. In Hellwing et al. (2018), heifers were fed
612 silages originating from the same experiment as Damborg et al. (2019), and they concluded that
613 CH_4 yield was higher when feeding pulp silage compared to the whole plant silage (30.9 vs. 29.2
614 L/kg DMI, respectively). In contrast to our study, Hellwing et al. (2018) found that the proportion
615 of acetate was lower and the proportion of butyrate was higher, when feeding pulp silage compared
616 to the corresponding grass-clover silage. To our knowledge, no other in vivo experiments
617 measuring production of CH_4 using lactating cows fed with pulp have been conducted.
618
619 **CONCLUSIONS**

Rumen digestibility of NDF and DNDF increased linearly, when LGS was substituted with L1×P and L2×P, respectively, whereas the protein value (g AA digested in the small intestine/kg of DMI) and the CH_4 yield (L/kg of DMI) increased, when EGS was substituted with E1×P and E2×P, respectively. Unexpectedly, k_d of DNDF did not increase but k_p of iNDF decreased when GS was substituted with 1×P and 2×P, respectively, and highlights a need for further investigation of the relation between physical processing and fiber kinetics in the rumen. The substitution of GS with 1×P and 2×P, respectively, displayed mostly linear responses, but for some variables, the response was quadratic. The results indicated that pulp silage of grass from green biorefining can be a valuable feedstuff for dairy cows, but the change in feeding value by the wet fractionation depends on the developmental stage of grass during harvest.

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Table 1. Chemical composition (g/kg of DM unless otherwise stated) of experimental silages and extracts[1] of these (n = 4).

Developmental stage	Early			Late			SEM[3]	*P*-values[2]				
Silage type	Grass	Pulp	Pulp	Grass	Pulp	Pulp		Dev	Processing			
Processing	Chopped	Fractionated once	Fractionated twice	Chopped	Fractionated once	Fractionated twice			L	Q	L × Dev	Q × Dev
DM, g/kg	266	272	390	200	296	377	3.2	<0.01	<0.01	<0.01	<0.01	<0.01
Ash	98.3	64.3	37.9	91.8	56.2	37.6	1.32	<0.01	<0.01	<0.01	0.03	0.06
OM	902	936	962	908	944	962	1.3	<0.01	<0.01	<0.01	0.03	0.06
NDF	452	527	669	487	593	692	7.7	<0.01	<0.01	0.04	0.45	0.02
iNDF[4], g/kg of NDF	83.9	95.3	98.8	109	102	99.7	3.37	<0.01	0.40	0.83	<0.01	0.28
iNDF	37.9	50.2	66.0	53.0	60.2	68.9	1.83	<0.01	<0.01	0.44	<0.01	0.76
Hemicellulose	198	215	314	201	254	314	5.2	<0.01	<0.01	<0.01	0.72	<0.01
ADF	254	312	355	286	339	378	3.0	<0.01	<0.01	0.01	0.19	0.91
Cellulose	242	297	337	268	319	358	3.4	<0.01	<0.01	0.04	0.54	0.82
ADL	12.1	15.5	17.7	18.1	20.3	19.8	1.63	0.01	0.04	0.50	0.25	0.79
CP	182	187	188	159	163	164	2.2	<0.01	0.02	0.40	0.87	0.85
Total AA	117	118	129	85.1	98.6	98.3	1.77	<0.01	<0.01	0.49	0.85	<0.01
N, g/kg of total N												
Soluble N	594	535	306	575	512	346	10.7	0.94	<0.01	<0.01	0.02	0.09
Ammonia N	36.3	40.2	39.4	43.8	39.4	41.4	8.24	0.68	0.97	0.95	0.74	0.70
AA-N	533	527	590	446	505	505	8.0	<0.01	<0.01	0.74	0.90	<0.01
OMD,[5] %	78.9	77.7	75.4	75.3	74.3	73.4	0.33	<0.01	<0.01	0.45	0.03	0.28
pH	4.18	3.99	3.96	4.07	3.95	3.97	0.018	<0.01	<0.01	<0.01	<0.01	0.84
Acetate	29.9	25.3	15.8	36.4	22.9	16.5	0.53	<0.01	<0.01	0.29	<0.01	<0.01
Propionate	1.75	3.56	2.42	4.84	3.33	2.58	0.410	0.01	0.07	0.14	<0.01	0.02
Caproate	0.140	0.185	0.127	0.243	0.0815	0.132	0.02868	0.95	0.05	0.30	0.11	0.01
L-lactate[6]	37.6	46.8	27.3	46.4	40.8	28.3	0.84	0.09	<0.01	<0.01	<0.01	<0.01
Glucose	1.52	1.62	1.31	0.957	1.11	1.06	0.2327	0.03	0.82	0.46	0.51	0.79
Buffer capacity[7]	26.2	20.7	16.7	19.2	19.5	16.2	0.54	<0.01	<0.01	0.28	<0.01	0.02

[1]Extracts were also analyzed for butyrate, but it was not detected.

[2]*P*-values: Dev = developmental stage; L = linear effect of intensifying fractionation; Q = quadratic effect of intensifying fractionation; L × Dev = interaction between the linear effect of intensifying fractionation and Dev; Q × Dev = interaction between the quadratic effect of intensifying fractionation and Dev.

[3]Higest standard error of the mean.

[4]Indigestible NDF.

[5]In vivo OM digestibility calculated as 4.10 + 0.959 × in vitro OM digestibility.

[6]L-lactate constitutes about half of total lactate (Johansen et al., 2020).

[7]meq/100 g DM

Table 2. Feed intake (kg/d unless otherwise stated) in cows fed TMR based on chopped grass and pulp silage of fractionated grass harvested at two developmental stages (n = 4).

Item	Treatment[1]						SEM[3]	*P*-values[2]				
	Early			Late				Dev	Processing			
	GS	1×P	2×P	GS	1×P	2×P			L	Q	L × Dev	Q × Dev
DM	17.8	16.3	15.1	17.2	16.3	14.7	0.67	0.43	<0.01	0.85	0.75	0.66
OM	16.2	15.2	14.3	15.7	15.2	14.0	0.62	0.51	<0.01	0.69	0.79	0.60
CP	3.25	3.02	2.79	2.86	2.75	2.50	0.123	<0.01	<0.01	0.66	0.60	0.64
NDF	5.86	6.09	7.00	5.97	6.85	7.15	0.238	0.02	<0.01	0.87	0.91	0.05
Hemicellulose	2.72	2.63	3.40	2.63	3.09	3.37	0.107	0.09	<0.01	0.02	0.70	<0.01
ADF	3.14	3.46	3.60	3.35	3.76	3.78	0.134	0.01	<0.01	0.11	0.91	0.58
Cellulose	2.97	3.27	3.39	3.11	3.52	3.56	0.134	0.04	<0.01	0.14	0.89	0.61
ADL	0.170	0.198	0.203	0.238	0.245	0.221	0.0166	<0.01	0.63	0.32	0.12	0.88
iNDF[4]	0.530	0.611	0.721	0.679	0.720	0.734	0.0352	<0.01	<0.01	0.99	0.03	0.60
Total AAN	0.305	0.282	0.278	0.247	0.253	0.229	0.0108	<0.01	0.01	0.69	0.55	0.10
Total AA	2.28	2.10	2.03	1.84	1.89	1.70	0.081	<0.01	<0.01	0.55	0.43	0.13
DM intake, % of BW	3.00	2.67	2.52	2.86	2.66	2.45	0.140	0.35	<0.01	0.58	0.74	0.58
NDF intake, % of BW	0.986	1.00	1.17	0.996	1.12	1.19	0.0531	0.10	<0.01	0.40	0.94	0.09

[1]Treatments: Early = grass harvested at early developmental stage; Late = grass harvested at early developmental stage; GS = Silage of chopped grass; 1×P = silage of pulp fractionated once; 2×P = silage of pulp fractionated twice.

[2]*P*-values: Dev = developmental stage; L = linear effect of substituting GS with 1×P and 2×P, respectively; Q = quadratic effect of substituting GS with 1×P and 2×P, respectively; L × Dev = interaction between the linear effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev; Q × Dev = interaction between the quadratic effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev.

[3]Higest standard error of the mean.

[4]Indigestible NDF.

Table 3. Duodenal flow and nutrient digestibility in cows fed TMR based on chopped grass and pulp silage of fractionated grass harvested at two developmental stages (n = 4 unless otherwise stated).

Item	Treatment[1]						SEM[3]	*P*-values[2]				
	Early			Late				Dev	Processing			
	GS	1×P	2×P	GS	1×P	2×P			L	Q	L × Dev	Q × Dev
Duodenal flow (g/kg DMI)												
Total CP	203	206	231	196	187	194	8.9	<0.01	0.03	0.06	0.01	0.79
Total AA	128	128	150	121	117	122	6.1	<0.01	<0.01	0.03	0.01	0.40
Microbial AA	55.8	53.0	54.7	56.1	46.2	46.5	2.39	0.01	0.02	0.07	0.08	0.49
Feed + endogenous AA	71.0	76.1	96.0	66.4	69.6	74.9	4.92	<0.01	<0.01	<0.01	<0.01	0.03
Apparent ruminal digestibility, %												
CP	-11.1	-11.4	-24.8	-17.7	-10.3	-14.0	5.24	0.55	0.17	0.05	0.02	0.87
NDF[4]	73.7	72.1	74.0	66.2	71.7	76.6	1.86	0.15	<0.01	0.59	<0.01	0.44
DNDF[4,5]	80.5	79.3	81.0	74.3	80.3	84.3	1.89	0.59	<0.01	0.86	<0.01	0.33
Hemicellulose[4]	66.6	62.9	70.1	55.2	64.8	72.5	2.52	0.12	<0.01	0.17	<0.01	0.06
Cellulose[4]	84.6	83.4	82.1	79.6	82.1	84.4	1.32	0.16	0.34	0.92	<0.01	0.98
True ruminal digestibility, %												
DM	48.0	46.2	42.0	45.6	46.0	45.7	2.95	0.76	0.06	0.54	0.05	0.73
OM	59.8	58.8	54.6	58.8	57.9	57.9	2.53	0.66	0.02	0.61	0.12	0.35
CP	36.9	33.4	21.1	33.5	32.5	28.4	3.89	0.38	<0.01	0.01	<0.01	0.25
Small intestinal digestibility, %												
OM[4]	58.7	60.2	54.8	53.3	54.2	55.2	2.79	<0.01	0.42	0.09	0.02	0.09
CP[4]	74.2	75.4	73.4	72.2	72.5	73.6	0.95	<0.01	0.65	0.23	0.08	0.06
AA[4]	76.8	78.8	78.8	76.8	77.8	78.6	1.11	0.56	0.05	0.49	0.92	0.61
Apparent total tract digestibility, %												
DM	77.6	77.8	73.9	75.7	75.0	76.1	0.88	0.17	0.03	0.34	0.01	0.02
OM	79.0	79.7	75.9	77.6	76.9	77.9	0.81	0.17	0.03	0.20	0.01	0.01
CP	72.2	73.8	69.4	70.2	70.6	72.1	0.88	0.23	0.59	0.09	0.01	0.01
NDF	76.0	77.0	76.5	71.5	74.0	78.4	1.29	0.03	<0.01	0.93	<0.01	0.38
DNDF[5]	83.1	84.6	84.0	80.1	82.9	86.2	1.17	0.30	<0.01	0.65	0.01	0.45
Hemicellulose	71.3	72.6	75.3	64.9	70.8	76.9	1.61	0.06	<0.01	0.71	0.01	0.81
Cellulose	85.0	85.4	82.4	81.5	82.2	84.0	1.06	0.02	0.99	0.46	<0.01	0.16

[1]Treatments: Early = grass harvested at early developmental stage; Late = grass harvested at early developmental stage; GS = Silage of chopped grass; 1×P = silage of pulp fractionated once; 2×P = silage of pulp fractionated twice.

[2]*P*-values: Dev = developmental stage; L = linear effect of substituting GS with 1×P and 2×P, respectively; Q = quadratic effect of substituting GS with 1×P and 2×P, respectively; L × Dev = interaction between the linear effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev; Q × Dev = interaction between the quadratic effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev.

[3]Higest standard error of the mean.

[4]n = 3 for; Early: GS, 1×P, and 2×P; Late: GS.

[5]Digestible NDF = NDF – indigestible NDF.

Table 4. Nutrient digestion and efficiency of microbial protein synthesis in cows fed TMR based on chopped grass and pulp silage of fractionated grass harvested at two developmental stages (n = 4 unless otherwise stated).

Item	Treatment[1]						SEM[3]	*P*-values[2]				
	Early			Late				Dev	Processing			
	GS	1×P	2×P	GS	1×P	2×P			L	Q	L × Dev	Q × Dev
Digested in the rumen, kg/d												
NDF[4]	4.30	4.61	5.28	3.95	4.94	5.52	0.166	0.50	<0.01	0.93	0.05	0.11
DNDF[4,5]	4.31	4.53	5.17	3.92	4.96	5.46	0.189	0.37	<0.01	0.81	0.03	0.07
Digested in the small intestine												
AA[4], g/d	1731	1786	1814	1499	1481	1423	121.3	<0.01	0.96	0.79	0.31	0.96
AA[4], g/kg of DMI	95.7	103	118	88.0	89.5	94.7	6.04	<0.01	<0.01	0.33	0.03	0.75
Efficiency of microbial CP synthesis[6]												
g CP/kg of OM	163	153	164	162	134	133	11.7	0.04	0.18	0.18	0.15	0.87
g CP/kg of NDF[4]	350	327	247	353	236	195	16.3	<0.01	<0.01	0.70	0.06	0.01

[1]Treatments: Early = grass harvested at early developmental stage; Late = grass harvested at early developmental stage; GS = Silage of chopped grass; 1×P = silage of pulp fractionated once; 2×P = silage of pulp fractionated twice.

[2]*P*-values: Dev = developmental stage; L = linear effect of substituting GS with 1×P and 2×P, respectively; Q = quadratic effect of substituting GS with 1×P and 2×P, respectively; L × Dev = interaction between the linear effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev; Q × Dev = interaction between the quadratic effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev.

[3]Higest standard error of the mean.

[4]n = 3 for; Early: GS, 1×P, and 2×P; Late: GS.

[5]Digestible NDF = NDF – indigestible NDF.

[6]Microbial CP synthesized (= duodenal flow of microbial CP) per kg of OM and NDF digested in the rumen.

Table 5. Rumen contents and rumen kinetics of fiber in cows fed TMR based on chopped grass and pulp silage of fractionated grass harvested at two developmental stages (n = 4).

Item	Treatment[1]						SEM[3]	*P*-values[2]				
	Early			Late				Dev	Processing			
	GS	1×P	2×P	GS	1×P	2×P			L	Q	L × Dev	Q × Dev
Total content, kg	86.4	96.1	105	91.6	99.9	108	6.72	0.19	<0.01	0.90	0.75	0.98
Free fluid, kg	27.3	26.9	25.2	24.6	30.1	25.3	2.36	0.88	0.69	0.06	0.41	0.14
Free fluid, % of total	32.0	28.0	24.3	27.0	29.8	23.4	1.96	0.22	<0.01	0.06	0.15	0.05
DM, g/kg	113	120	123	115	117	125	2.9	0.98	<0.01	0.94	1.00	0.27
Pools, kg												
DM	9.79	11.5	12.8	10.5	11.7	13.3	0.74	0.15	<0.01	0.99	0.85	0.56
OM	8.82	10.4	11.6	9.47	10.7	12.2	0.674	0.11	<0.01	0.99	0.92	0.57
NDF	4.93	5.99	6.92	5.49	6.16	7.26	0.482	0.08	<0.01	0.72	0.66	0.50
iNDF[4]	1.23	1.79	1.81	1.70	1.85	2.05	0.122	<0.01	<0.01	0.09	0.18	0.05
DNDF[5]	3.69	4.21	5.10	3.80	4.31	5.21	0.389	0.53	<0.01	0.28	0.98	0.98
Rates (%/h)												
K_d DNDF[6]	5.42	4.76	4.46	4.79	4.97	4.43	0.537	0.60	0.06	0.75	0.39	0.36
K_p iNDF[7]	1.70	1.33	1.52	1.62	1.64	1.36	0.133	0.78	0.05	0.47	0.70	0.03

[1]Treatments: Early = grass harvested at early developmental stage; Late = grass harvested at early developmental stage; GS = Silage of chopped grass; 1×P = silage of pulp fractionated once; 2×P = silage of pulp fractionated twice.

[2]*P*-values: Dev = developmental stage; L = linear effect of substituting GS with 1×P and 2×P, respectively; Q = quadratic effect of substituting GS with 1×P and 2×P, respectively; L × Dev = interaction between the linear effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev; Q × Dev = interaction between the quadratic effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev.

[3]Higest standard error of the mean.

[4]Indigestible NDF.

[5]Digestible NDF = NDF – iNDF.

[6]Fractional rate of digestion of DNDF in the rumen.

[7]Fractional rate of passage of iNDF out of the rumen.

Table 6. Rumen fluid pH and composition in cows fed TMR based on chopped grass and pulp silage of fractionated grass harvested at two developmental stages (n = 4).

Item	Treatment[1]						SEM[3]	*P*-values[2]				
	Early			Late				Dev	Processing			
	GS	1×P	2×P	GS	1×P	2×P			L	Q	L × Dev	Q × Dev
pH	6.38	6.32	6.44	6.40	6.31	6.34	0.045	0.90	1.00	0.24	0.50	1.00
Total SCFA[4], mmol/L	129	134	125	129	138	132	4.6	0.32	1.00	0.06	0.47	0.96
SCFA proportions, mol per 100 mol of total SCFA												
L-lactate[5]	0.273	0.233	0.101	0.224	0.338	0.147	0.0748	0.58	0.09	0.12	0.52	0.40
Acetate	61.5	63.4	66.5	62.8	63.9	67.0	0.61	0.08	<0.01	0.10	0.43	0.68
Propionate	20.4	20.8	20.3	19.3	20.0	18.6	0.75	0.02	0.59	0.15	0.63	0.63
Isobutyrate	1.03	1.05	0.937	0.945	0.976	0.908	0.0421	<0.01	0.01	<0.01	0.26	0.60
Butyrate	12.7	10.5	8.86	12.6	11.1	10.1	0.603	0.13	<0.01	0.45	0.18	0.99
Isovalerate	2.06	2.19	1.91	2.07	2.10	1.95	0.243	0.90	0.29	0.17	0.88	0.60
Valerate	1.69	1.35	1.09	1.41	1.25	1.05	0.068	<0.01	<0.01	0.65	<0.01	0.30
Caproate	0.396	0.387	0.225	0.562	0.402	0.300	0.0438	<0.01	<0.01	0.24	0.06	0.01
Acetate:propionate	3.04	3.04	3.31	3.27	3.22	3.63	0.145	0.01	0.01	0.08	0.68	0.66
Ammonia N, mmol/L	7.95	9.63	5.33	6.68	8.77	6.72	0.941	0.69	0.10	<0.01	0.09	0.49
Glucose, mmol/L	0.974	0.674	0.490	0.663	0.531	0.513	0.1079	0.08	<0.01	0.50	0.10	0.99

[1]Treatments: Early = grass harvested at early developmental stage; Late = grass harvested at early developmental stage; GS = Silage of chopped grass; 1×P = silage of pulp fractionated once; 2×P = silage of pulp fractionated twice.

[2]*P*-values: Dev = developmental stage; L = linear effect of substituting GS with 1×P and 2×P, respectively; Q = quadratic effect of substituting GS with 1×P and 2×P, respectively; L × Dev = interaction between the linear effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev; Q × Dev = interaction between the quadratic effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev.

[3]Higest standard error of the mean.

[4]Short-chained fatty acids.

[5]L-lactate constitutes about half of total lactate (Johansen et al., 2020).

Table 7. Milk production in cows fed TMR based on chopped grass and pulp silage of fractionated grass harvested at two developmental stages (n = 4).

Item	Treatment[1]						SEM[3]	*P*-values[2]				
	Early			Late				Dev	Processing			
	GS	1×P	2×P	GS	1×P	2×P			L	Q	L × Dev	Q × Dev
Yield												
Milk, kg	25.4	25.0	24.1	24.3	24.4	21.0	1.97	0.20	0.14	0.47	0.53	0.56
ECM, kg	25.1	24.2	24.2	24.3	24.3	21.4	1.78	0.31	0.18	0.68	0.50	0.41
Fat, g	1026	970	982	1000	1013	900	70.5	0.61	0.17	0.74	0.60	0.28
Protein, g	857	838	839	819	797	717	66.6	0.14	0.29	0.85	0.46	0.68
Lactose, g	1164	1138	1117	1118	1136	958	99.2	0.26	0.18	0.46	0.46	0.45
Composition, %												
Fat	4.07	3.95	4.10	4.17	4.15	4.29	0.192	0.04	0.48	0.20	0.66	0.76
Protein	3.43	3.39	3.47	3.37	3.29	3.43	0.141	0.03	0.23	0.02	0.79	0.54
Lactose	4.56	4.58	4.62	4.58	4.64	4.56	0.110	0.65	0.45	0.20	0.20	0.12
NUE[4], %	25.5	27.2	29.2	28.1	28.4	28.0	1.47	0.37	0.14	0.92	0.11	0.80
Kg ECM/kg DMI	1.38	1.48	1.60	1.41	1.51	1.46	0.076	0.46	<0.01	0.36	0.09	0.27

[1]Treatments: Early = grass harvested at early developmental stage; Late = grass harvested at early developmental stage; GS = Silage of chopped grass; 1×P = silage of pulp fractionated once; 2×P = silage of pulp fractionated twice.

[2]*P*-values: Dev = developmental stage; L = linear effect of substituting GS with 1×P and 2×P, respectively; Q = quadratic effect of substituting GS with 1×P and 2×P, respectively; L × Dev = interaction between the linear effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev; Q × Dev = interaction between the quadratic effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev.

[3]Higest standard error of the mean.

[4]Nitrogen use efficiency = milk N as % of N intake.

Table 8. Gas exchange in cows fed TMR based on chopped grass and pulp silage of fractionated grass harvested at two developmental stages (n = 4).

Item	Treatment[1]						SEM[3]	*P*-values[2]				
	Early			Late				Dev	Processing			
	GS	1×P	2×P	GS	1×P	2×P			L	Q	L × Dev	Q × Dev
CH_4												
L/d	591	588	555	639	611	577	28.3	0.04	0.01	0.58	0.51	0.71
L/kg of DMI[4]	32.3	34.3	35.1	37.3	37.0	36.8	1.24	<0.01	0.07	0.62	0.01	0.56
L/kg of ECM[4]	23.8	24.2	22.3	26.3	25.4	25.9	1.58	<0.01	0.22	0.71	0.47	0.14
CO_2, L/d	6676	6383	5979	6574	6270	5765	221.0	0.22	<0.01	0.52	0.70	0.86
CH_4:CO_2	0.0883	0.0917	0.0926	0.0972	0.0973	0.100	0.00216	<0.01	0.01	0.95	0.64	0.23
O_2, L/d	6023	5706	5352	5828	5583	5095	188.7	0.03	<0.01	0.45	0.78	0.59
H_2, L/d	3.79	2.52	2.71	4.18	2.46	2.25	0.371	0.86	<0.01	<0.01	0.15	0.97
RQ[5]	1.11	1.12	1.12	1.13	1.12	1.13	0.014	0.04	0.36	0.72	0.62	0.49

[1]Treatments: Early = grass harvested at early developmental stage; Late = grass harvested at early developmental stage; GS = Silage of chopped grass; 1×P = silage of pulp fractionated once; 2×P = silage of pulp fractionated twice.

[2]*P*-values: Dev = developmental stage; L = linear effect of substituting GS with 1×P and 2×P, respectively; Q = quadratic effect of substituting GS with 1×P and 2×P, respectively; L × Dev = interaction between the linear effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev; Q × Dev = interaction between the quadratic effect of substituting GS with 1×P and 2×P, respectively, and the effect of Dev.

[3]Higest standard error of the mean.

[4]DMI and ECM yield determined in the period, where gas exchange was measured, and therefore differs from results reported in Table 2.

[5]Respiration quotient = CO_2 produced divided by O_2 consumed.

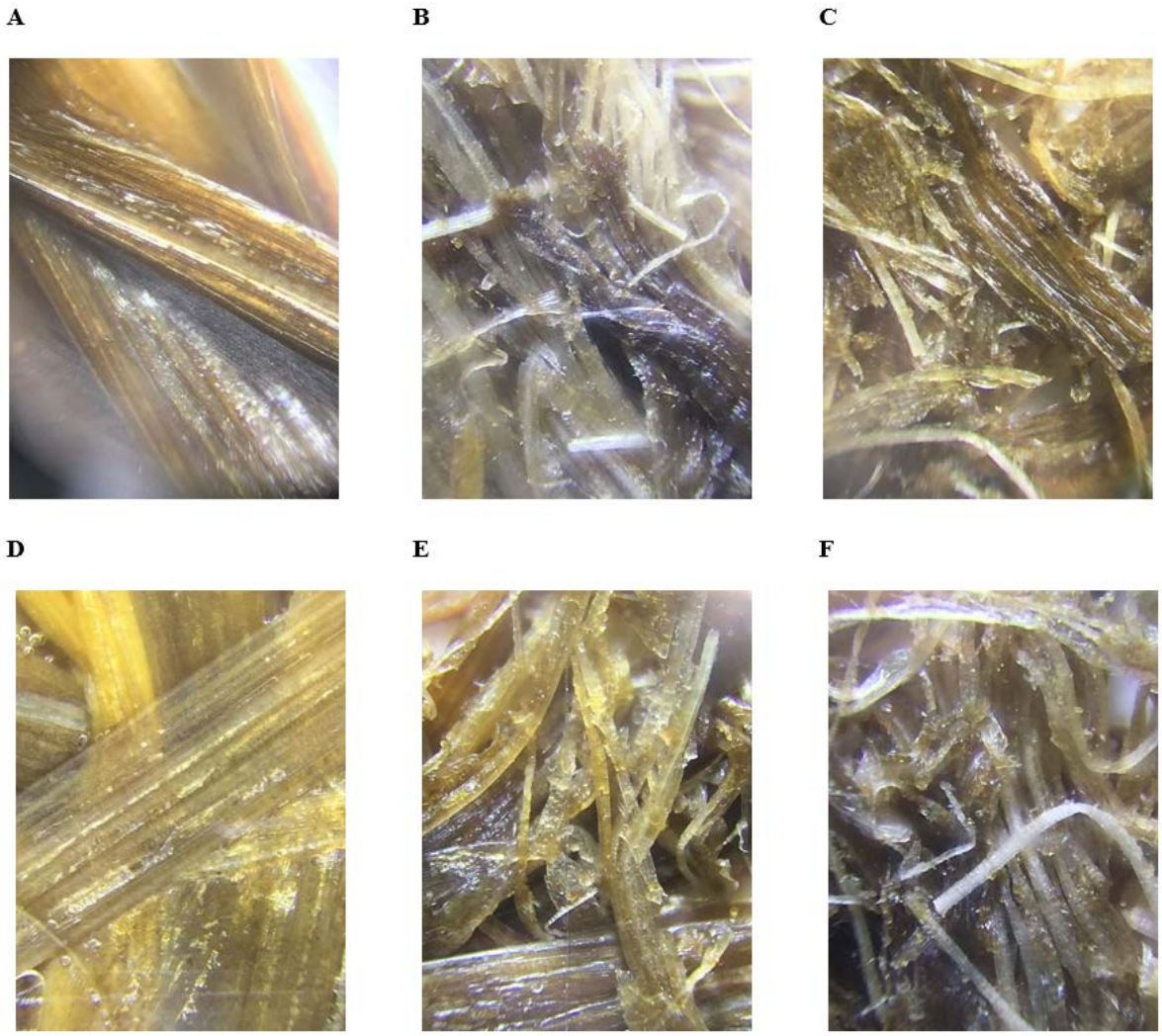

Figure 1. Pictures (125 × magnification using microscope) of silage particles from A) early harvested grass (EGS), B) pulp fractionated once from early harvested grass (E1×P), C) pulp fractionated twice from early harvested grass (E2×P), D) late harvested grass (LGS), E) pulp fractionated once from late harvested grass (L1×P), and F) pulp fractionated twice from late harvested grass (L2×P).

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6 General discussion

The overall aim was to investigate the effects of feeding grass harvested at different developmental stages as either fresh grass or as silage from physically processed grass on mainly neutral detergent fibre (**NDF**) digestibility, feed intake, methane emission, and milk production. Those aspects were analysed in the three studies (the Fresh-Study, the Shred-Study, and the Pulp-Study) and addressed and discussed in detail in Papers I-V, whereas in this chapter, they are discussed in a broader context and in relation to practical farming.

6.1 Changes in nutrient composition and digestibility of grass during its development

On practical farms, the applied cutting strategy of green forages depends on the relationships between requirements for feed quality and quantity and the costs associated with cutting, transport, and storage. Increasing the number of cuts, and thereby minimising the length of the regrowth period between cuts, will improve organic matter (**OM**) digestibility (**OMD**), but at the same time reduce herbage yield within a cut (Weisbjerg et al., 2010). Consequently, an increased number of cuts per year will also increase the machinery operation costs, suggesting that the optimal number of cuts depends on the marginal response in milk production to improved forage OMD and, although not discussed further in this thesis, to the price relations between inputs (final price of the forage) and milk. As argued by Johansen (2017), the optimum OMD of forages regarding milk production is probably in the range of 80-82%, since the effects of increased OMD on milk production are still positive in this range. However, the diminishing effect of OMD starts at a lower level and the economic optimum might be at a different level (Jensen et al., 2015, Daniel et al., 2016). Achieving such high OMD of grass-clover could be acquired by cutting more frequently as investigated in the three studies of this thesis. However, the aim of this PhD project was also to investigate the combined effects of processing and harvesting forage at late developmental stage to increase field yield while maintaining high OMD by means of physical processing (discussed in section 6.2). Together, the three studies illuminate the effects of plant development on forage chemical composition, digestibility, and feed intake as discussed below.

Chemical composition

Generally, the consensus is that with increased developmental stage at harvest, the stem proportion, NDF concentration, and indigestible NDF (**iNDF**) proportion have all increased, whereas crude protein (**CP**) concentration and OMD have decreased (Wilson, 1993, 1994). Figure 6.1 illustrates the effects of length of regrowth on those parameters of forages analysed in the three studies of this PhD project. The stem proportion on DM basis in the Fresh-Study changed throughout the feeding study, whereas the difference between grass harvested at early and late developmental stages remained similar. Therefore, Figure 6.1 shows the average stem proportion determined throughout Fresh-Study, and across the three studies, stem proportion varied from 9 to 56% and increased between 0.2 and 1.5 %-units per day. The daily decrease in CP concentration was between 2.1 and 4.5 g CP/kg of DM, which resembles the average daily decrease of 3.0 g CP/kg of DM reported by Rinne et al. (1997). The greater decrease in

CP concentration for the Shred-Study compared to the Fresh-Study and Pulp-Study might partly be caused by the greater daily increase in stem proportion, as CP concentration is generally higher in leaves than stems (Mowat et al., 1965). Per day, the NDF concentration increased between 2.6 and 8.7 g NDF/kg of DM and iNDF proportion increased between 0.1 and 8.5 g iNDF/kg of NDF. The greatest daily increases of NDF and iNDF were observed for forage in the Shred-Study, which could be explained by the higher proportion of clover and greater increase in stem proportion (Wilson and Kennedy, 1996). Moreover, perennial ryegrass was the only grass species in the Fresh-Study and Pulp-Study, whereas the grass in the Shred-Study was constituted of both perennial ryegrass and festulolium of variety Fojtan. The festulolium of variety Fojtan resembles tall fescue more than Italian ryegrass, which could probably explain the greater daily increase in NDF concentration and iNDF proportion (Østrem et al., 2015). However, the variation in chemical composition related to progressing developmental stage often exceeds that of variation between and within cool-season grasses (Buxton and Marten, 1989). The daily reduction in OMD varied from 2.1 to 9.2 g/kg of OM, and mirrored the daily changes in NDF concentration, and stem and iNDF proportion. For the reported variables in Figure 6.1, the change with progressing days for regrowth will likely follow at curve-linear trend rather than the reported linear trend based on two points (Rinne, 2000), and the development might also be affected by environment-related factors such as cumulative temperature, rainfall, etc. (Wilson, 1994, Buxton, 1996).

Digestion and feed intake

In the Shred-Study and Pulp-Study, the fractional rate of digestion (**k_d**) of digestible NDF (**DNDF**) was determined using the rumen evacuation technique and the degradation rate of DNDF was determined by in situ incubation. The results of the in situ incubation of silages from the Pulp-Study are reported in Bitsch (2021). Increased developmental stage resulted in a daily decrease in the degradation rate of DNDF ranging from 0.01 to 0.13 %-units/h, when determined in situ, whereas k_d of DNDF determined from rumen incubations resulted in daily changes ranging from +0.02 to -0.11 %-units/h. The two methods might yield different results, since the in situ method determines the degradation rate of DNDF of individual feedstuffs, whereas using rumen evacuations determines the k_d of DNDF of the total diet (Huhtanen et al., 2007a). However, grass-clover was the sole feedstuff in the Shred-Study. Kuoppala et al. (2010) found decreases in the k_d of DNDF of similar altitude with progressing developmental stage. The rates of decrease in k_d of DNDF of forages in the Shred-Study and the control silage in the Pulp-Study were similar. Despite the stem proportion of forage in the Shred-Study was 3-5 times higher than the forage in the Pulp-Study, the daily increase in ADL concentration was similar and was likely caused by the high temperatures during the development of forage in the Pulp-Study (Buxton, 1996). Moreover, the k_d of DNDF is higher in clover than in grass (Wilson and Kennedy, 1996), possibly counteracting the effect of higher stem proportion on changes in k_d of DNDF for forage in the Shred-Study compared to forage in the Pulp-Study.

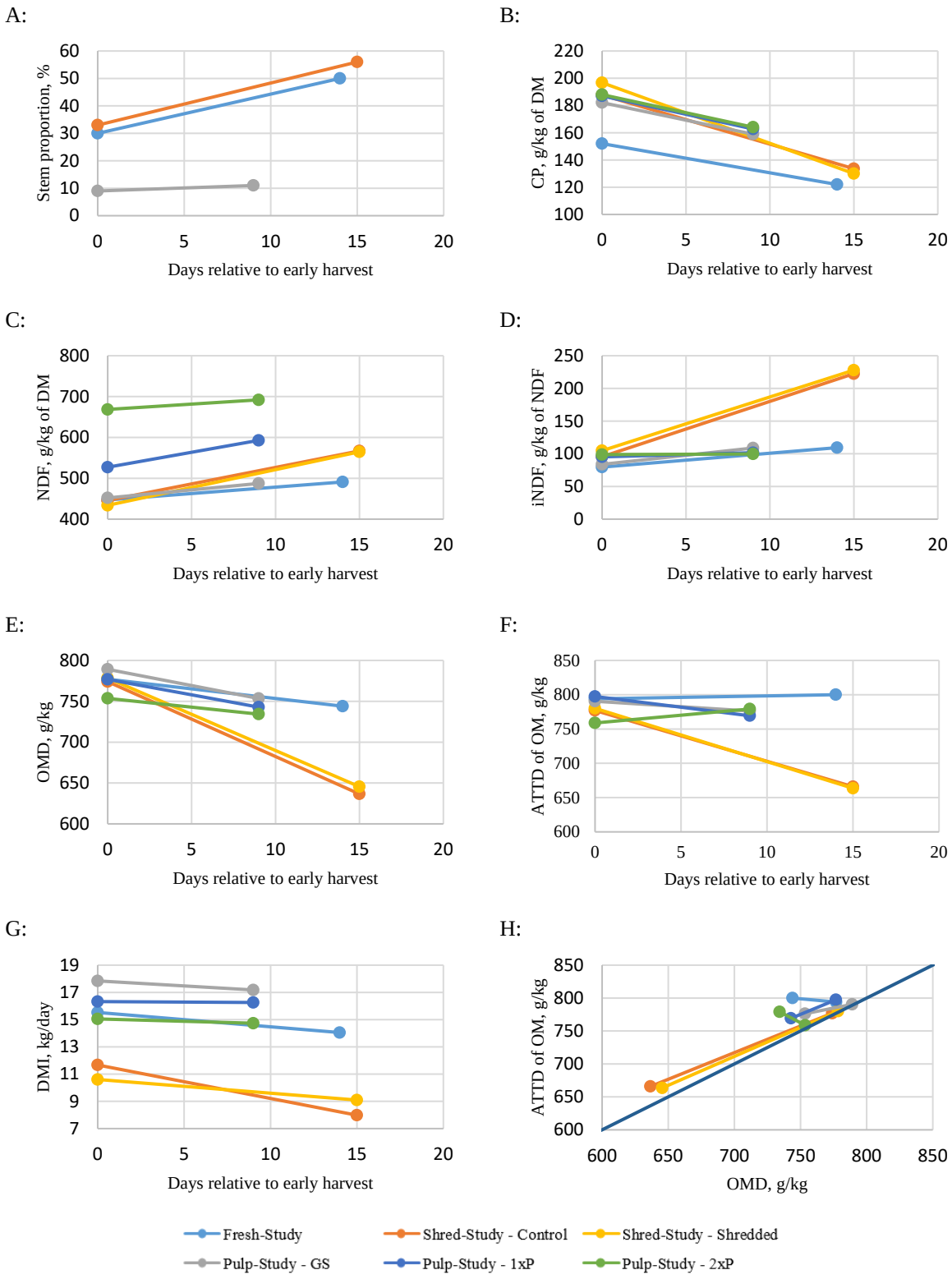

Figure 6.1: Relationships between characteristics of forages and the developmental stage of forage at harvest (x-axis; days of growth relative to cut at early developmental stage) in the three feeding studies. A) stem proportion, B) crude protein (CP) concentration, C) neutral detergent fibre (NDF) concentration, D) proportion of indigestible NDF, E) in vitro determined organic matter (OM) digestibility (OMD), F) apparent total tract digestibility (ATTD) of OM, and G) dry matter (DM) intake (DMI). H) Comparison of ATTD of OM and OMD.

The daily change in apparent total tract digestibility (**ATTD**) of OM with progressing development of forages ranged from -7.8 to +2.3 g/kg, and especially for silages in the Pulp-Study, ATTD of OM seemed to be higher compared to the in vitro determined OMD. Normally, concentrate supplementation increases DMI, resulting in lower digestibility compared to situations where forages are fed at levels closer to the maintenance level (Moorby et al., 2006). There was no clear explanation for this unexpected effect for silages in the Pulp-Study, although similar observations were reported in Johansen et al. (2017b). For every 1 %-unit decrease in OMD, the decrease in DMI ranged from 0.02 to 0.45 kg/day and averaged 0.2 kg/day. The response was similar to the 0.2 kg/day decrease in DMI for every 1 %-unit decrease in the D-value (digestible OM in DM) reported by Pang et al. (2019). Compared to the two other experiments, the markedly lower OMD of silages harvested at late compared to early developmental stages in the Shred-Study did not result in a proportionally similar decrease in DMI. This may have been due to differences in the passage rates, since the iNDF proportion increased more with an extended length of the regrowth period in the Shred-Study compared to the Fresh-Study and Pulp-Study.

6.2 Effects of physical processing and interactions with developmental stage

As already implied, forage OMD can negatively correlated with the herbage yield in the field, but also determines the response in energy-corrected milk (**ECM**) yield (Jensen et al., 2015; Daniel et al., 2016). Several methods of physical processing have been studied with the aims of improving nutrient digestibility, and if improvement in digestibility is achieved, this method in combination with postponed harvest could result in higher herbage yields of digestible nutrients. Of all nutrients, OM and NDF digestibility have garnered the most attention when assessing the effects of physical processing. Independent of the response variable in question (e.g. ruminal digestion kinetics, ECM yield), the reported effects of physical processing are equivocal, possibly because the effects also depend on many other characteristics related to the forage (e.g. forage type, stem proportion, developmental stage of forage at harvest) and processing technique (e.g. method, intensity) as discussed in the following sections.

Chemical composition and in vitro digestibility

With shredding, no nutrients or plant fractions are removed or fractionated from the forage. However, shredding of forages with low DM concentrations can result in losses of nutrients through effluents while the forage is left for wilting in very humid or rainy conditions (Savoie, 2001). Moreover, loss of nutrients and DM might also be caused by loss of plant particles while handling the material in the field (McGechan, 1989). Loss of particles in grass-clover could also hamper the nutritional quality since the loss of mainly clover leaves would decrease CP concentration and increase NDF concentration (McGechan, 1989). In the Shred-Study, DM losses were not estimated, but the differences between shredded and non-shredded grass-clover would have been small since the forages were treated similarly until the time point where it was either baled directly (non-shredded) or shredded and then baled. In the Shred-Study, shredded grass-clover was loaded directly from the shredder to a wagon and transported to a bunker silo where it was baled, thereby minimizing the risk of losing plant particles. The CP concentration of the shredded and non-shredded silages in the Shred-Study indicated that loss

of clover leaves was avoided. The shredder used in the Shred-Study was a prototype, and the future design of the machine will probably be a combined shredder and baler.

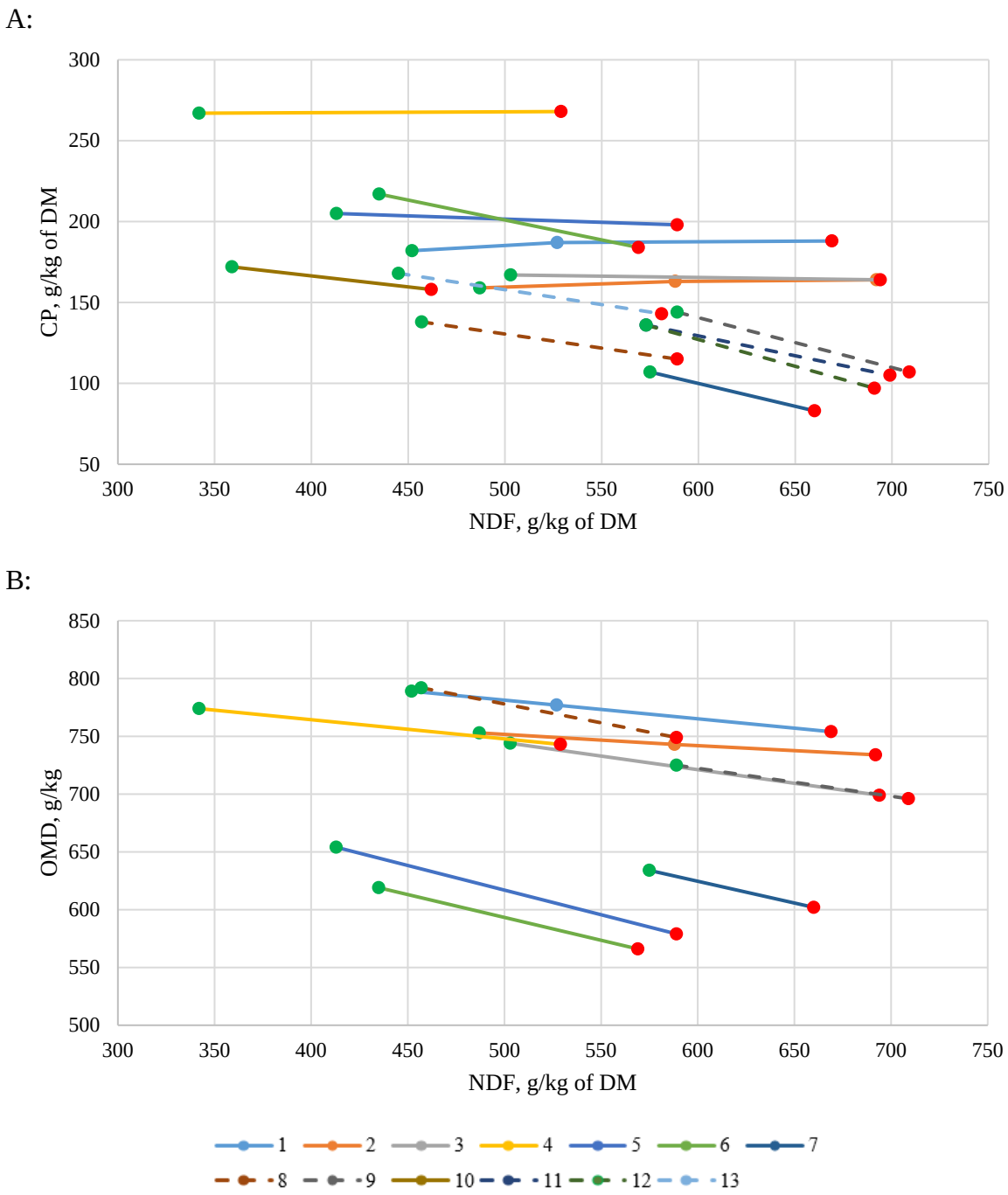

Figure 6.2: Relationship between A) crude protein (CP) and neutral detergent fibre (NDF) concentration and B) in vitro determined organic matter digestibility (OMD) and NDF concentration in whole plant (green dot) and pulp of the whole plant (red dot). Bold lines indicate fresh forages was fractionated and dashed lines indicate silage was fractionated. Data: 1) Shred-Study (Early), 2) Shred-Study (Late), 3) Damborg et al. (2018; perennial ryegrass), 4) Damborg et al. (2018; white clover), 5) Damborg et al. (2018; red clover), 6) Damborg et al. (2018; lucerne), 7) Larsen et al. (2019), 8) Sousa et al. (2022), 9) Savonen et al. (2019), 10) Santamaria-Fernandez et al. (2019), 11) Piou et al. (2020; Haarslev), 12) Piou et al. (2020; Angel), 13) Rinne et al. (2020).

Figure 6.2 compares results from the Pulp-Study (Paper V) and other recent literature where fresh and ensiled forages have been fractionated. As shown in Figure 6.2, the

concentration of NDF and CP and the in vitro determined OMD of pulp are different from the whole crop it came from. Pulp of fresh forage and silage has been shown to have a chemical composition, which in most cases will be suitable for ruminant nutrition. The data in Figure 6.2 indicate that NDF concentration is on average 140 g/kg of DM higher in pulp compared to the whole crop, but the difference ranges from 75 to 217 g NDF/kg of DM. The minimum and maximum differences between the whole plant and pulp were obtained in the Pulp-Study, suggesting that the Pulp-Study reflected the extremes in terms of efficiency of extraction of nutrients during fractionation; hence, extreme responses of animal performance could be expected in this feeding study. Despite that the change was not significant, the reduction in ECM yield when feeding cows with pulp pressed twice compared to the whole plant (2×P vs. GS in the Pulp-Study) was indeed larger than the difference shown in other studies with pulp (Damborg et al., 2019; Savonen et al., 2019; Sousa et al., 2022).

In practical farming, it might be undesirable if the CP concentration in the pulp becomes much lower than the whole plant it came from. The data in Figure 6.2 show that CP concentration in pulp from fresh forage is between 24 g/kg of DM lower and 6 g/kg of DM higher compared to the whole plant. In contrast, work focusing on fractionation of forage that has already been ensiled reported only lower CP concentrations in pulp compared to the whole crop (between 23 and 39 g CP/kg of DM lower). Various reasons lie behind the decision of fractionating either fresh forages or ensiled forages. In a meta-analysis, Franco et al. (2019) reported that additive application and harvest (primary vs. regrowth) had little effect on liquid yield when fractionating silages. Franco et al. (2019) did not report results of the true protein proportion in the liquid fraction from the silage, which, due to hydrolysis of peptide bonds and deamination of AA during ensiling, might be lower in the liquid fraction when fractionating silage compared to fresh forage. The higher proportion of soluble N in silage compared to the fresh forage might be the reason why the CP concentration of the pulp fraction from fractionation of silage compared to fresh forage is generally lower (Figure 6.2). Except for the type of forage that was fractionated (fresh vs. ensiled), it is difficult to relate the difference in CP concentration between pulp and the whole crop to other factors, such as forage type, developmental stage of forage at harvest, concentration of other nutrients, etc. Further research should emphasise describing and quantifying this variation. In the Pulp-Study, it was suggested that CP concentration was higher in pulp compared to the whole plant due to extraction of also other nutrients than CP into the liquid fraction, which was indicated by the lower concentration of ash in pulp compared to the whole plant. Performing mass balances of soluble nutrients during fractionation, as done by Damborg et al. (2020), could improve our understanding of these differences between experiments. Based on Figure 6.2, the CP concentration is likely to be lower in pulp compared to the whole plant, which must be taken into account when assessing screw pressing compared to shredding as a method for physical processing in practice.

With the extraction of soluble nutrients and increased NDF concentration, the in vitro determined OMD would, accordingly, also be lower in pulp compared to the whole crop. Data in Figure 6.2 show that the OMD was on average 35 g/kg lower in pulp compared to the whole crop and the difference varied from -10 to -75 g/kg. For some of the experiments, ATTD of OM was also determined in vivo, which in some cases was higher for pulp compared to the whole crop despite the in vitro determined OMD showing otherwise. The mismatch between the in vitro method used for feedstuff evaluation and the in vivo determined OMD was addressed in Paper V and pointed out by Damborg et al. (2019). Farmers rely on rapid, reliable,

and cheap estimates of the quality of feedstuffs (pulp in this case); thus, if pulp becomes a future feedstuff, the method of determining its digestibility (in vitro determined OMD) needs to be revisited. The ATTD of OM and OMD determined in vitro in the Shred-Study seemed not to be affected by the physical processing. However, if more intensive shredding of forage is achieved using another machine, similar concerns might arise.

Compaction

The degree of physical processing of forages in the Shred-Study and the Pulp-Study were assessed in terms of their relative ability to be compressed. Compared to their respective control silages, the density (kg DM/m^3) of shredded grass, pulp pressed once, and pulp pressed twice were, across developmental stages, 37, 30, and 63% higher, respectively. Using the same design as the shredder in the Shred-Study, but on a smaller scale, Samarasinghe et al. (2019) increased density 45% when shredding perennial ryegrass four times. This could suggest that physical processing was more intense in the study of Samarasinghe et al. (2019) compared to the Shred-Study. Besides minimizing the requirement for storage capacity, the increased density from physical processing suggests that an anaerobic environment is established faster during the initial fermentation phase since oxygen removal is positively correlated with the level of compaction (Wilkinson and Davies, 2013; Samarasinghe et al., 2019). Moreover, the decline in pH is faster in shredded forages, probably due to a more available proportion of sugars and due to more destroyed cells in shredded forages, which promote a higher growth rate of lactic acid bacteria that produce fermentation acids (Savoie, 2001). However, controlling DM concentration at the onset of the ensiling process and the air infiltration still seem to have a greater influence on the ensiling characteristics than compaction (McEniry et al., 2007). Moreover, the effect of physical processing in the Pulp-Study on density was confounded with the concomitant decrease in buffer capacity. Using compaction or density as an explanatory variable for the degree of physical processing might be questionable based on the results obtained in the Shred-Study and Pulp-Study. Indeed, density was higher for physically processed forages in both studies, but the correlation to response parameters such as k_d of DNDF and digestibility of NDF was vague, as discussed in the following section.

Digestibility and DMI

The animal responses to physical processing in the Shred-Study and the Pulp-Study probably occurred for different reasons, since forages in the Shred-Study were only shredded (without removal of nutrients), whereas pulp distinguished from control silage in the Pulp-Study by having a higher concentration of NDF and by being more disintegrated, as indicated by Figure 1 in Paper V. Therefore, effects of structural changes of particles might be expected for both shredded forage and pulp, whereas additional effects of the increased NDF concentration might be expected primarily when feeding pulp. Moreover, due to the fractionation using a screw press, pulp also has a lower concentration of water-soluble compounds such as sugars and non-protein N (**NPN**), and the proportion of true protein in CP is higher since most of the soluble NPN has been extracted (Damborg et al., 2018; Damborg et al., 2020). However, based on in situ determination, the k_d of DNDF of experimental silages in the Shred-Study (Paper III) and the Pulp-Study (Bitsch, 2021) were not affected by physical processing. Neither were the k_d of DNDF determined based on the rumen evacuation technique

in the Shred-Study (Paper III) or the Pulp-Study (Paper V). Moreover, physical treatment had no effect on the fractional rate of passage ($\mathbf{k_p}$) of iNDF in the Shred-Study and decreased k_p of iNDF linearly in the Pulp-Study. The combined effects of k_d and k_p resulted in similar ruminal digestibility of NDF but lower ATTD of NDF in the Shred-Study. Processing forage harvested at late developmental stage in the Pulp-Study resulted in higher ruminal and total tract digestibility of NDF, probably due to the decreased k_p. Similar to the outcomes in the Shred-Study and the Pulp-Study, results reported in the literature are equivocal regarding effects of physical processing on digestibility, probably due to the inconsistent use of animal species (cows, steers, goats, sheep), forage type (grass, lucerne, grass-clover), and type of machine for the physical processing. Moreover, sufficient information regarding whether or the degree to which the forage has been treated sufficiently is most often lacking.

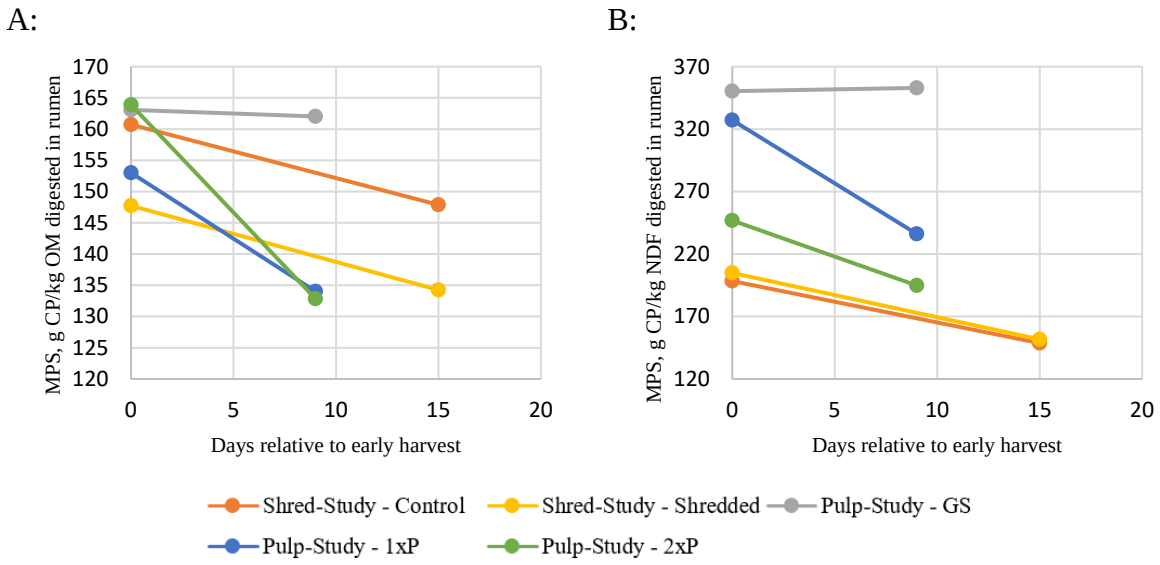

Figure 6.3: Relationship between the efficiency of microbial protein synthesis (MPS; expressed in relation to A) OM and B) NDF digested in the rumen) and the developmental stage of forage at harvest (x-axis; days of growth relative to cut at early developmental stage) in cows fed forages from the Shred-Study and Pulp-Study.

The efficiency of the microbial protein synthesis in the rumen, expressed as g microbial CP synthesised per kg of either OM or NDF digested in the rumen, was generally lower, when feeding forage harvested at late compared to early developmental stage as shown in Figure 6.3. Although low DMI suppresses the efficiency of the microbial protein synthesis (Archimède et al., 1997), the lower DMI observed for forages harvested at late developmental stage in the Shred-Study and the Pulp-Study could not be the sole explanation for the reduction in efficiency. The main cause of the reduced efficiency was not clear. However, the change in nutrient composition of microbes in the Shred-Study suggested that the change in nutrient composition of the diets altered the bacterial community (Belanche et al., 2012). In the feed evaluation system NorFor (Volden, 2011), the response in the efficiency of the microbial protein synthesis to intake level of starch and residual carbohydrates is curve-linear, but not to such a degree, that it can explain the effects of either developmental stage at harvest or the effect of physical processing in the Shred-Study and the Pulp-Study. Furthermore, the k_p was higher for grass-clover harvested at late compared to early developmental stage in the

Shred-Study, whereas no effect on k_p of developmental stage at harvest was observed in the Pulp-Study. The differences in k_p might have caused a shift in the microbial population and thereby the relatively larger decrease in efficiency (g microbial CP/kg of OM truly digested in the rumen) in the Shred-Study compared to the Pulp-Study.

As shown in Figure 6.4, there seemed to be a high correlation between the rumen pool size of NDF and DMI within each study. The data suggest that the cows did not eat to obtain a certain pool size (kg) of NDF in the rumen. The changes in DMI might be attributed to the consistency of the rumen content, which (by visual inspection) seemed to change with the intensity of physical processing in the Pulp-Study. This structural change might have had different effects on the rumen load and caused different stimuli to rumen stretch receptors. However, the structural change was also confounded with increasing NDF concentration in the pulp compared to the chopped grass, which most likely limited the DMI of cows fed pulp compared to chopped grass. In practical farming, it seems that pulp has to substitute not only traditionally chopped grass to avoid rations having too high concentrations of NDF.

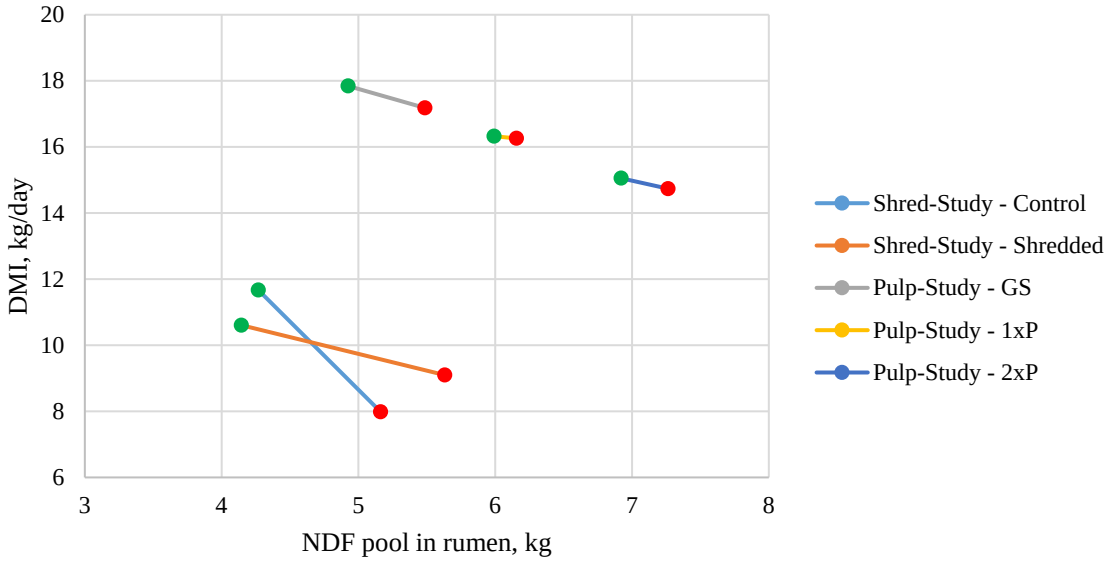

Figure 6.4: Relationship between dry matter intake (DMI) and neutral detergent fibre (NDF) pool in the rumen of cows fed forages in the Shred-Study and the Pulp-study, grouped after type of treatment and indicated as forage harvested at either early (green dot) or late developmental stage (red dot).

6.3 Methane emission

Improvement of forage digestibility has been suggested as a potential approach to mitigate the enteric production of CH_4 from dairy cows (Boadi et al., 2004; Brask et al., 2013). Since the effect of shredding on ruminal digestibility of OM and especially NDF have shown both negative and positive effects throughout literature, a large variation in effects of shredding on CH_4 yield (L/kg of DMI) is also expected. Improving digestibility due to higher k_d of DNDF would enable more readily fermentable substrates for fermentation in the rumen, but also higher intake resulting in reduced CH_4 yield (L/kg of DMI). Despite that the particle structure of pulp is also more disintegrated compared to the chopped silage of the whole plant, the effect of physical processing cannot be expected to be the same as for shredding, due to the much higher NDF concentration in the pulp compared to the chopped silage of the whole plant. The

increased NDF concentration will favour the formation of acetic acid and thereby the production of CH_4, and combined with lower DMI when feeding pulp, the CH_4 yield (L/kg DMI) would increase. However, the CH_4 yield was not different between the pulp and control treatments in the Pulp-Study, when forage harvested at late developmental stage was processed.

A total of 14 dietary treatment means were obtained for CH_4 production (L/day) and CH_4 yield (L/kg of DMI) in the three studies of this thesis (Figure 6.5), of which 8 treatments included concentrate supplementation (34 and 35% on DM-basis of total DMI in the Fresh-Study and the Pulp-Study, respectively) and no concentrates were fed in the remaining treatments. Despite CH_4 production and yield increased with increasing DMI, there was no clear distinction in CH_4 emission between rations with concentrate supplementation, and those without, as would have been expected (Olijhoek et al., 2018). Instead, the distinction seemed to be attributed to the three experiments themselves.

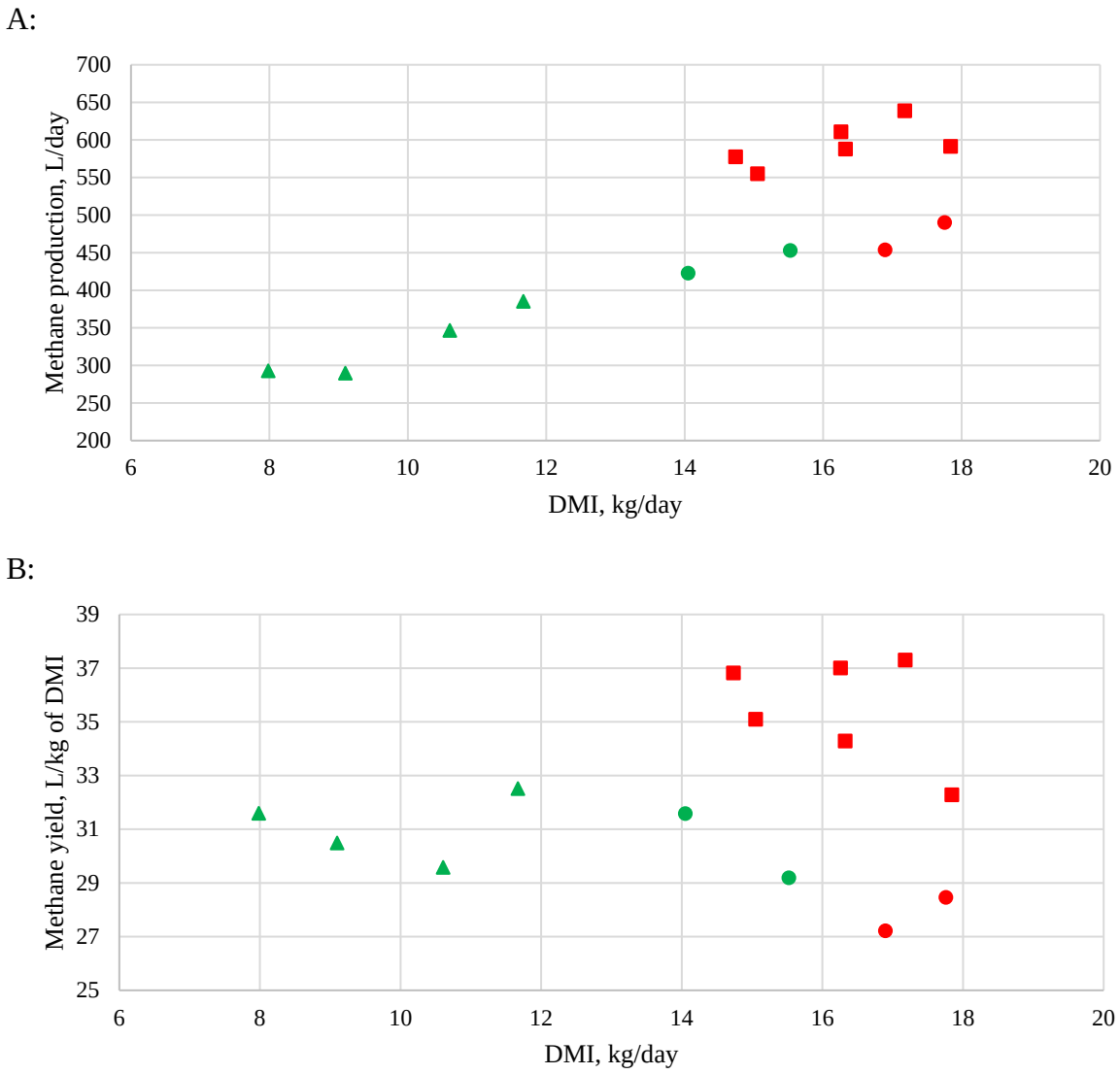

Figure 6.5: Relationship between A) methane production, B) methane yield and dry matter intake (DMI) for cows in the Fresh-Study (circles), Shred-Study (triangles), and Pulp-Study (squares) either supplemented with concentrates (red dots) or not (green dots)

In the concept of biorefining, the change in the enteric CH_4 emission coupled to ruminant nutrition should be assessed comparatively. This means that the net production of CO_2-equivalents in a traditional crop rotation for a dairy farm with typically high inclusion of maize for silage in Denmark should be compared to the alternative crop rotation, in which most of the maize has been substituted by grass or grass-clover. Moreover, the alternative crop rotation suited for biorefining production seeks to substitute imported protein (e.g. soybean meal), which requires the whole supply chain to be included in a life cycle assessment. However, performing such a life cycle assessment was beyond the scope of this thesis.

6.4 Milk production in cows fed high proportions of grass

Mainly the Fresh-Study aimed at investigating the effects of dietary treatments on milk yield and milk composition, whereas, although milk yield and overall composition were investigated in both the Shred-Study and the Pulp-Study, the main objective was to investigate the effects of physical processing on nutrient digestibility. Especially for the Shred-Study, the cows were in late lactation and low yielding as shown in Figure 6.6. Moreover, the cows in all three studies generally achieved DMI that was lower than expected based on previous studies investigating effects of similar diets and following similar procedures for measuring digestibility (Johansen et al., 2017b; Weisbjerg et al., 2018; Kragbæk Damborg et al., 2019). Figure 6.6 indicates that there was a close correlation between DMI and ECM yield, and across studies, cows produced 1.4 kg ECM/kg of DMI. Within the studies, there was no clear relationship between the OMD and ECM yield or between ATTD of OM and ECM yield and there was no clear reason for the generally low DMI.

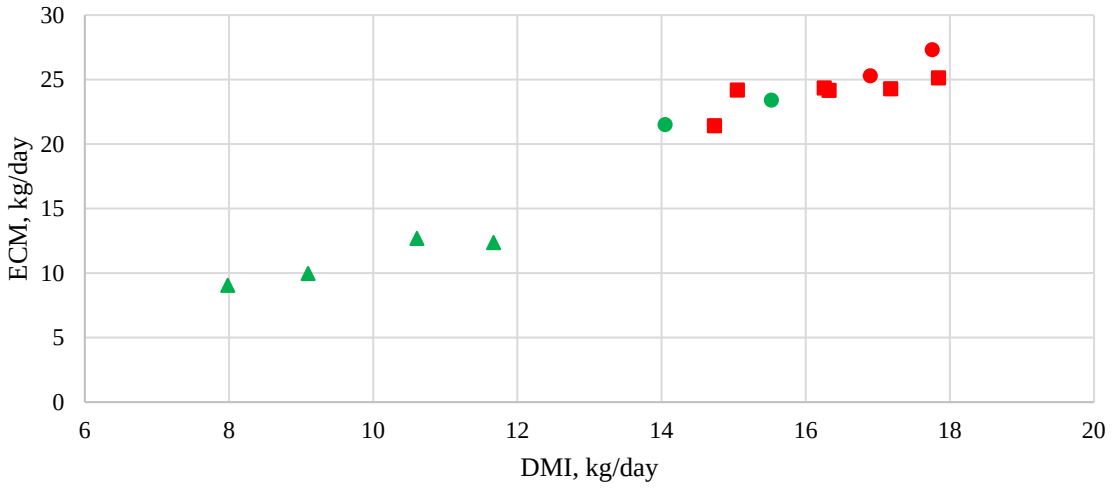

Figure 6.6: Relationship between energy-corrected milk (ECM) yield and dry matter intake (DMI) for cows in the Fresh-Study (circles), Shred-Study (triangles), and Pulp-Study (squares) either supplemented with concentrates (red dots) or not (green dots).

The level of ECM yield ranged from 21.4 to 27.3 kg/day in the Fresh-Study and the Pulp-Study and resembles several different feeding regimes through which milk can be produced (i.e., nearly 100% forage vs. concentrate supplementation, silage vs. fresh forage feeding, feeding forage harvested at early vs. late developmental stage, and feeding

traditionally chopped silage or pulp from a biorefinery). Feeding nearly 100% forage has given rise to special dairy products such as “GRASSMILK”, where lower yields (as shown in the Fresh-Study) are expected since the animals are fed forage as the sole feedstuff. The concept requires that the farmers receive a premium for the milk but will leave more land for production of grains and vegetables for human consumption. The Fresh-Study investigated the effect of feeding fresh grass in the barn because it, among other things, would solve some logistic problems on large farms such as long walking distances to distant fields (Van den Pol-van Dasselar et al., 2008). Intake and utilization of fresh grass can also be manipulated by using different grazing systems, but assessing these systems was beyond the scope of this thesis.

On Danish practical farms, grass is mostly sown in mixtures with clover, which increases the forage CP concentration (Van Keuren and Hoveland, 1985), and the mixtures contain several types of grass species. The effect of clover proportion or grass species could not be assessed in the three studies of this thesis, since these factors did not differ within each study. Based on previous literature, feeding legume-based diets compared to grass-based diets generally increases DMI and milk yield (Hoffman et al., 1998) but decreases the concentration of protein and fat in the milk. However, at similar OM digestibility, DMI and milk yield were similar within family (Johansen et al., 2017a). This suggests, that if OMD of silage and fresh grass harvested at late developmental stage in the Fresh-Study had not differed by 49 g/kg, the DMI and milk yield of cows fed fresh grass had been more similar to that of the cows fed silage. However, the numerically lower DMI and milk yield in cows fed the fresh grass compared to silage in the Fresh-Study was probably also caused by longer particles of the fresh grass compared to precision chopped silage (Nasrollahi et al., 2015).

7 Conclusion

This thesis contributes with improved knowledge on digestion, feed intake, milk production, and methane production when feeding high proportions of grass harvested at different developmental stages as either fresh or as silage from physically processed grass. Based on the Fresh-Study, it was concluded that milk yield was higher in cows fed silage compared to harvested fresh grass fed in the barn, probably due to higher ATTD of OM. However, despite numeric differences, there was no difference in DMI or ECM yield between cows fed silage compared to harvested fresh grass fed in the barn.

The effects of physical processing of forage prior to ensiling were investigated in two studies by either comparing shredded to un-shredded grass-clover (the Shred-Study) or by comparing pulp of fractionated grass to traditionally chopped grass (the Pulp-Study). It was concluded that physical processing improved total tract and ruminal digestibility of NDF, when comparing pulp of grass harvested at late developmental stage to chopped grass harvested at late developmental stage. When comparing pulp of grass harvested at early developmental stage to chopped grass harvested at early developmental stage, no effects were observed for NDF digestibility, whereas shredding decreased total tract digestibility of NDF. Shredding had no effect on DMI, whereas cows fed pulp had lower DMI but higher NDF intake compared to cows fed chopped grass.

The effects on the production of methane of feeding grass or grass-clover harvested at an early and late developmental stage were investigated in all three studies. Based on the Fresh-Study and the Shred-Study, it was concluded that feeding grass or grass-clover varying in the developmental stage at harvest had no effect on methane yield (L/kg of DMI). However, in the Shred-Study, cows fed grass-clover harvested at early developmental stage or fed shredded grass-clover decreased the production of methane expressed as L/kg of OM digested in the rumen. In the Pulp-Study, the effects on methane yield of the developmental stage of grass at harvest interacted with the effects of physical processing. This means that the methane yield was higher for cows fed grass harvested at late compared to early developmental stage when grass was chopped, whereas the difference in methane yield between cows fed grass harvested at late compared to early developmental stage was smaller when fed as pulp.

8 Perspectives

There are certain areas that would benefit from further research to optimise the production of biomasses for biorefineries and to quantify the environmental effects of fully implementing the concept of biorefinery as an industrial sector.

From the dairy farmer's perspective, the inclusion of pulp in the diets for the dairy cows, or alternatively heifers and dried-off cows, presents numerous challenges that must be addressed. However, the challenges depend on the time perspective, as the current commercial production of pulp in Denmark is limited to two facilities. Increasing the amount of pulp produced by increasing the number of biorefineries will overcome some of the challenges occurring in Danish settings as mentioned below, merely due to the advantages of scale of production.

To enlighten the economic potential of biorefineries in Denmark, Jørgensen et al. (2021) concluded that current scenarios of protein production for organic or non-GM production systems would be feasible. Logistically, a central biorefinery has the capacity to process green biomass from approximately 2,500 ha assuming biomass is processed non-stop in the growing season. Considering 65% of DM from the whole crop is recovered in the pulp (Damborg et al., 2020), a forage yield of 10 tons of DM/ha, and the biorefinery is running for 6 months, the amount of pulp produced per day reaches approximately 90 tons pulp DM. The research included in the Pulp-Study supports the fact that pulp ensiles very well compared to the whole crop, also without application of silage additives. However, practically, daily production of 90 tons pulp DM is equal to a harvest of approximately 30 ha/day in the first cut of grass-clover with traditional mowing, wilting, and precision chopping. In many cases, present day bunker silos at dairy farms are dimensioned for much larger amounts, highlighting the need for increasing the scale of production to optimise the logistics during ensiling of the pulp. In several studies, pulp has been ensiled with success using different methods such as 500 g vacuum bags (Paper IV), 200 L air tight drums (Paper V), and in +1000 kg wrapped round bales (Kragbæk Damborg et al., 2019). This suggests that alternative methods might be utilised for the conservation of pulp, possibly also with success.

The challenge with the dimension of the biorefinery can be met, if silage is also fractionated, and, in addition, silage can be fractionated all year utilizing the full capacity of the biorefinery. If the liquid fraction is intended to be used directly on farm rather than processing it into a protein concentrate with multiple side-streams, usage of a smaller screw press (1 ton per hour) could be used on-farm, by feeding pulp directly to the dairy cows. However, the protein in liquid from fractionated silage can probably not be precipitated and extracted to similar degree as for the liquid from fractionation of fresh grass. The liquid from fractionation of silage therefore should therefore be fed to animals close to the production site to avoid high transportation costs of large quantities of water. It could be attractive to feed pulp from fractionation of fresh forage directly to dairy cows and thereby overcome the challenge with sparse amounts of pulp to ensile at a time. However, studies that feed pulp of fresh forage directly to the dairy cows has yet to come, and the value of protein (i.e. true protein proportion) must be assessed as well.

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