GREEN BIOREFINING OF GRASSLAND BIOMASS

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Green biorefining of grassland biomass

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Data sheet

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Preface

This report is an update of DCA Report No 93 from 2017 on protein production from green biomass. The Ministry of Food, Agriculture and Fisheries has requested the update because both research and commercial activities in this area develop rapidly, and new knowledge is continuously produced. The purpose of the report is to summarize our present knowledge on the bio-technical as well as economic issues in relation to value creation of green biomass in Denmark. This includes many types of knowledge from different research areas along the production chain, and therefore researchers from several departments at Aarhus University as well as from Dept. of Food and Ressource Economics, Copenhagen University have contributed.

Contents

Summary

Utilization of 'green biomass' for producing high quality proteins has been proposed as a mean to substitute other protein sources for monogastric animals and humans, and at the same time obtain environmental benefits when the production of green biomass substitutes production of maize, cereals or other annual crops. The aim of this report is to summarize our present knowledge on the bio-technical as well as economic issues in relation to value creation of green biomass in Denmark. The report focuses on the resource base for producing and obtaining green biomass, the environmental impacts related to the production hereof, the concepts for biorefining, the quality of the products produced and possible business cases.

Considering availability and quality of green biomass, grasses and grass-clover crops grown in rotation on arable land shows a huge potential to deliver high yields of biomass as well as protein with an appropriate amino acid profile. For pure grasses, the protein yield increases significantly with increased N fertilization without impairing protein quality. In grass-clover mixtures the importance of N fertilization is much lower. New initiatives on plant breeding to increase production and in particular protein production or persistence are going on, but the outcome of these initiatives is yet not clear. Grass from unfertilized permanent grassland may represent an opportunity if focus is on the fibre part of the grass. However, if focus is on the protein part, it is required that the permanent grass is fertilized with nitrogen, which in some cases may counteract other environmental issues. For cover crops to be an attractive supply of biomass new production systems need to be developed, e.g. by an earlier harvest of the main crop and use of legume cover crop species, or by fertilizing non-legumes in order to have a sufficiently high production to cover harvesting costs.

There is clear evidence that changing from winter wheat or maize to either grass-clover or fertilized pure grass result in a decreased N-leaching and decreased greenhouse gas emissions, when the difference in soil carbon storage is taken into account. Only in a situation with very high N-fertilization to longer lasting grass field these benefits may disappear or become less pronounced. The environmental benefit of using permanent wet grassland for production remains to be documented.

It is estimated that by the present technology for biorefining, 40% of the protein present in the green biomass can be recovered in a protein concentrate having protein content in the range of 50% of dry matter (DM), similar to the protein content of soybean meal. Higher concentrations are possible to produce as well for specialty applications. In addition, a fibre fraction containing 15-18% protein in DM can be produced and used for ruminant feed, bioenergy production or even further biorefined into chemical building blocks or used for bio-materials such as food packaging.

Based on laboratory assessments, the protein concentrate is expected to be able to replace traditional protein sources for monogastrics, like pigs and poultry. The potential is confirmed by several animal experiments, where soy was replaced, either partially or completely, without negative effects on animal performance. High contents of unsaturated fat in the protein affect the meat and fat tissue and may be a limiting

factor for the amount of included protein. Based on the chemical composition and on feeding trials, the fibre fraction seems suitable for ruminant feeding replacing other types of silages.

Currently, the first industrial scale biorefineries of green biomass for feed and bioenergy are established in Denmark. Furthermore, it is investigated how the protein can be used directly for human consumption. In this respect, more fundamental and applied research is needed to evaluate the protein quality for food applications. Although promising results are available with respect to food functionality and thereby applicability as food ingredients, more knowledge is needed. In addition, a full European Food Safety Authority (EFSA) assessment is requested for approval of protein from green biomass for human consumption.

There are major uncertainties in the economic assessment of establishing a full-scale bio-refinery based on the concepts mentioned above. Major obstacles are transportation costs and uncertainty in running cost for the biorefinery. It will be important that the energy use in the refinery is supported by renewable energy, some of which could be produced from the residuals such as anaerobic digestion of the residual liquid and/or some of the fibre fraction.

The largest prospects are currently within the organic sector where there is a need for locally sourced, sustainable protein sources. It is estimated that there are options to produce feed protein based on green biomass to cover the full protein requirements for the Danish organic pig and poultry sector.

A range of initiatives are now taking place as private-public co-operation in Denmark and other European countries in order to optimize the biorefinery concept, to develop more products and to reduce costs of operation. In addition, work to establish firmer documentation of the effects of grass and clover production on nitrate leaching, greenhouse gas emission and other environmental aspects is on-going, with the aim of being able to include these externalities economically or in policy.

Sammendrag

Udnyttelse af "grøn biomasse" til at producere højkvalitetsproteiner har været foreslået som et middel til at erstatte andre proteinkilder til monogastriske dyr og mennesker, der på samme tid kan sikre miljømæssige fordele, når produktionen af grøn biomasse erstatter produktionen af majs, korn eller andre etårige afgrøder. Formålet med denne rapport er at sammenfatte vores nuværende viden om biotekniske og økonomiske problemstillinger i forhold til at skabe værdi omkring udnyttelsen af grøn biomasse i Danmark. Rapporten fokuserer på ressourcebasen for produktion af grøn biomasse, den miljømæssige indflydelse under selve produktionen, begreber for bioraffinering, produktkvalitet og mulige business cases.

Når man ser på forsyningsmuligheder og kvaliteten af den grønne biomasse, viser dyrkning af rene græsser og kløvergræsblandinger, som dyrkes på omdriftsjord, et stort potentiale for at kunne levere store udbytter af biomasse og protein med en hensigtsmæssig aminosyreprofil. Når det drejer sig om rent græs, stiger proteinudbyttet signifikant med forhøjet N-gødskning uden at forringe proteinkvaliteten. I græskløverblandinger er vigtigheden af N-gødskningen betydeligt lavere. Nye initiativer for at øge produktionen og i særdeleshed proteinproduktionen samt græsmarksafgrødernes holdbarhed er i fuld gang, men resultaterne er endnu ikke klar. Græs fra ugødede, permanente græsmarker kan måske vise sig at være en mulighed, hvis der er fokus på fiberdelen i græs. Men hvis fokus er på proteindelen, kræver det, at det permanente græs gødes med kvælstof, hvilket kan påvirke andre miljømæssige forhold negativt. For at efterafgrøder skal kunne betragtes som en attraktiv forsyning af biomasse, skal der udvikles et nyt produktionssystem, f.eks. ved at høste hovedafgrøden tidligere og bruge bælgplanter som efterafgrøder, eller ved at gødske ikke-bælgplanter for at opnå en tilstrækkelig høj produktion til at dække høstomkostningerne.

Der er tydelig evidens for, at hvis man skifter fra vinterhvede eller majs til enten kløvergræs eller rent græs, resulterer det i reduceret N-udvaskning og reducerede drivhusgasemissioner – når man tager forskellen i jordens kulstoflager i betragtning. Kun i et scenarie med meget høj N-gødskning til længerevarende græsmarker vil disse fordele forsvinde eller blive mindre udtalte. Den miljømæssige fordel ved at bruge permanente, våde græsmarker til produktionsformål mangler fortsat at blive dokumenteret.

Det estimeres, at med den nuværende bioraffineringsteknologi vil 40% af proteinet i den grønne biomasse kunne blive udvundet i et proteinkoncentrat med et proteinindhold på omkring 50% af tørstof, hvilket svarer til proteinindholdet i sojakager. Det er muligt at opnå højere koncentrationer til mere specielle anvendelsesmuligheder. Desuden kan produceres en fiberfraktion, som typisk indeholder 15-18% protein i tørstof, og den kan bruges som foder til drøvtyggere, til produktion af bioenergi, til videre bioraffinering til kemiske byggeklodser, eller det kan bruges til biomaterialer til emballage i fødevareindustrien.

Baseret på laboratorieevalueringer forventes proteinkoncentratet at kunne erstatte traditionelle proteinkilder til monogastriske dyr så som svin og fjerkræ. Potentialet er bekræftet ved adskillige dyreforsøg, hvor soja er blevet erstattet, enten delvist eller fuldstændigt uden negative virkninger på dyrenes ydelser. Højt indhold af umættet fedt i det grønne protein har indflydelse på bindevævet i kød og fedt og kan have en begrænsende indvirkning på mængden af optaget protein. Baseret på den kemiske sammensætning og på fodringsforsøg ser det ud til, at fiberfraktionen er velegnet som foder til drøvtyggere og kan erstatte andre typer ensilage.

På nuværende tidspunkt er de første bioraffinaderier af grøn biomasse til foder og bioenergi i industriel skala i fuld gang med at blive etableret i Danmark. Dertil undersøges det også, om proteinet kan anvendes direkte i fødevarer. I denne henseende er det nødvendigt med yderligere fundamental og anvendt forskning for at kunne evaluere proteinkvaliteten til direkte human anvendelse. Selv om der allerede er lovende resultater, når det drejer sig om fødevarefunktionalitet og dermed anvendelighed i fødevarer, er det nødvendigt med mere viden. Ydermere er det nødvendigt med en fuldstændig European Food Safety Authority (EFSA) vurdering, for at protein fra grøn biomasse kan bruges direkte til human ernæring.

Der er store økonomiske usikkerheder forbundet med etablering af et fuldskalabioraffinaderi baseret på ovennævnte koncept. Der vil være store udfordringer omkring transport og logistik, samt usikkerhed omkring løbende udgifter til selve bioraffinaderiet. Det vil være vigtigt, at energiforbruget i raffinaderiet fortrinsvis kommer fra vedvarende energi, og noget af den vedvarende energi kan produceres som biogas fra restaffald og/eller fra fiberfraktionen.

De største muligheder findes p.t. inden for den økologiske sektor, hvor der er et behov for lokalt fremstillede og bæredygtige proteinkilder. Det vurderes, at det vil være muligt at producere foderprotein baseret på grøn biomasse til at dække hele proteinbehovet til den danske, økologiske grise- og fjerkræssektor.

Forskellige initiativer er allerede i gang i form af private og offentlige samarbejder i Danmark og andre europæiske lande omkring optimering af bioraffinaderikonceptet, for at kunne udvikle flere produkter og for at reducere produktionsomkostningerne. Ydermere arbejdes der fortsat på at producere konkret dokumentation omkring effekterne af græs- og kløverproduktion på N-udvaskning, drivhusgasudledning og andre miljøaspekter med henblik på at kunne inkludere disse eksternaliteter enten økonomisk eller politisk.

1 Introduction

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In 2017 the Danish Centre for Food and Agriculture published the report 'Green biomass - protein production through bio-refining' high-lightening the perspectives on producing high quality feed proteins from green biomass to substitute other protein sources for monogastric animals and for human consumption (Hermansen et al., 2017). Subsequently, the National Bioeconomy Panel published their recommendations on new value chains based on green biomass, and the need for a broad update and evaluation of the present concepts and experiences on value creation based on green biomass (National Bioeconomy Panel, 2018). At Aarhus University a large demonstration green biorefinery was inaugurated in 2019, kick-starting work on the upscaling of grass biorefinery technologies to come close to market conditions. The first commercial biorefineries were built in 2020 and 2021 and will now try to develop the first real business cases on green biorefining.

The idea of utilizing leaf-protein-concentrates as a protein source for animal or human consumption is not new but dates back to early 20th century where pioneering efforts led to significant amounts of research and pilot scale development (Pirie, 1942). Throughout the 20th century and well into the 21st there has been multiple attempts and supporting research to facilitate commercial success of green biorefineries in Denmark (Pedersen et al., 1979) and internationally (Chiesa and Gnansounou, 2011; Houseman and Connell, 1976; Näsi and Kiiskinen, 1985; Pirie, 1978; Pisulewska et al., 1991). However, these early evaluations did not value the environmental benefits by changing cropping systems, utilizing surplus grasslands and substituting imports of soy products from other continents with high carbon footprints. Such environmental effects have attained much more political focus over the past decades and their improvement is stipulated in national and EU legislation such as the Water Framwork Directive, Nitrate Directive, and the EU and Danish Climate policies. This combination of techno-economic and environmental potential supplemented with the inclusion of resent developments in biorefinery techniques in order to develop and document win-win solutions with good business economy, environmental benefits, no or negative iLUC, and improved self-sufficiency of protein concentrates is the main innovation of the concept.

The development of new crop production systems combined with green biorefineries is not just about technical development of the production circle. It is also important to discuss our total land-use in Denmark in relation to public wishes, environment, climate, and biodiversity. This discussion has been supported by several land-use and technology scenarios in Gylling et al. (2016), Larsen et al. (2017) and Mortensen & Jørgensen (2021). They show that the bioeconomy may contribute significantly to additional reductions in nitrate leaching and greenhouse gas (GHG) emissions, but the extent of the reductions depends a lot on the way agriculture is combined with the biobased energy and material sector. They also pinpoint that the development of the landscape in directions of either sustainable intensification, or towards extensification and a higher share of nature, are important determinants for the potential size of the bioeconomy and the reductions in emissions.

The aim of this report is to summarize our present knowledge on the ongoing biotechnical as well as economic research and development in relation to value creation of green biomass in Denmark. We have focused on the resource base for producing and obtaining green biomass, the environmental impacts related to the production hereof, and the concepts for biorefining, the potential product output as well as the quality of the products produced.

We limit the considerations to green biomass in the form of grasses and legumes harvested before maturity, where it is the vegetative parts of the biomass that are used for further value creation. Nonetheless, the technology may also be applied for any other green leaves from e.g. beet roots (Pirie, 1978).

2 Availability and quality of green biomass

Johannes Ravn Jørgensen[1] (2.1 + 2.2), Chiara De Notaris[1] and Esben Øster Mortensen[1] (2.3), Claudia Nielsen[1] (2.4 + 2.5), Torben Asp[2] (2.6)

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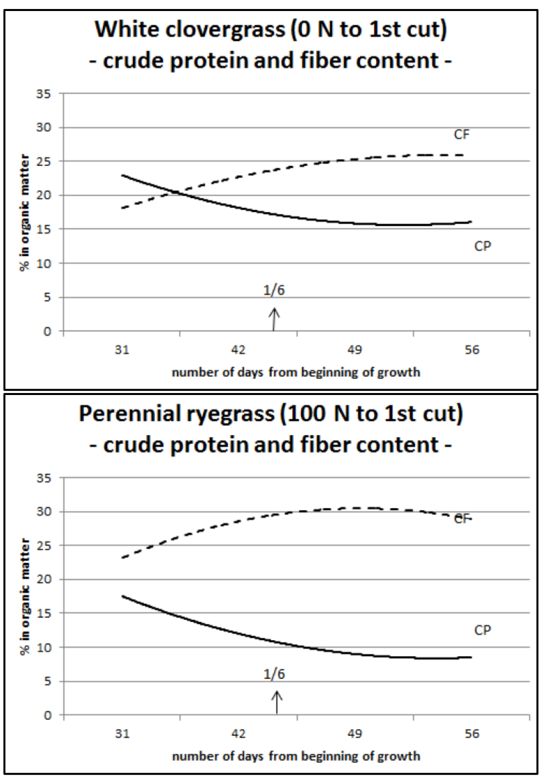
2.1 Characteristics of green biomass of importance for biorefining

The chemical composition of green biomass changes significantly depending on the maturity of the vegetation in grasses and clover. In early development stages grass leaves and clover leaves and petioles are the main constituent. In later development stages grass stem and leaf sheats and clover flowers and flower stems are the main constituents. The fibre content in DM increases while protein content decrease with increasing stage of development of plants. The changes are most pronounced in the beginning of the growth season. Figure 2.1 shows examples for white clover and grass.



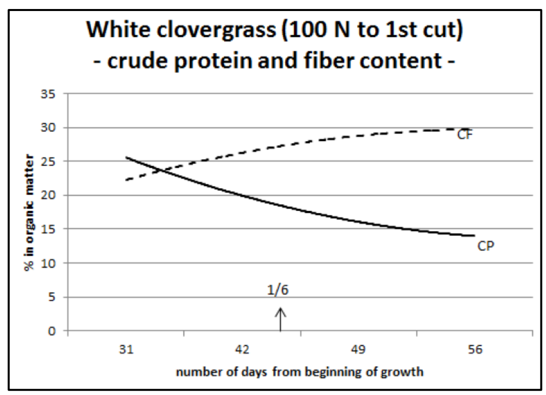


Figure 2.1. Changes in crude protein (CP) and crude fibre (CF) content by increased maturity of rye grass and grass-white clover with no N-fertilizer or fertilized with 100 kg N at the beginning of the growth season (after Pedersen and Møller, 1976).

The chemical composition and in particular the protein content depends on N fertilization. In Figure 2.2 is shown an example on the combined effect of N-fertilization and number of cuts (more cuts mean harvested at an earlier development stage) on biomass and protein yields over an entire season.

It appears that yield of biomass over an entire season does not depend very much on number of cuts, though three cuts typically yield the highest biomass. Likewise crude protein yield does not vary much dependent on number of cuts although it tends to be higher with five cuts in highly fertilized perennial ryegrass compared to three cuts. Also, while total protein yield is not influenced very much by N- fertilization in grass-clover mixtures, the yield of protein in ryegrass is very much increasing following increased N-fertilization. Thus, the protein to carbohydrate ratio is high in grasses that are cut frequently and supplemented with N fertilizer, while protein content in grass-clover only varies a little depending on N fertilization.

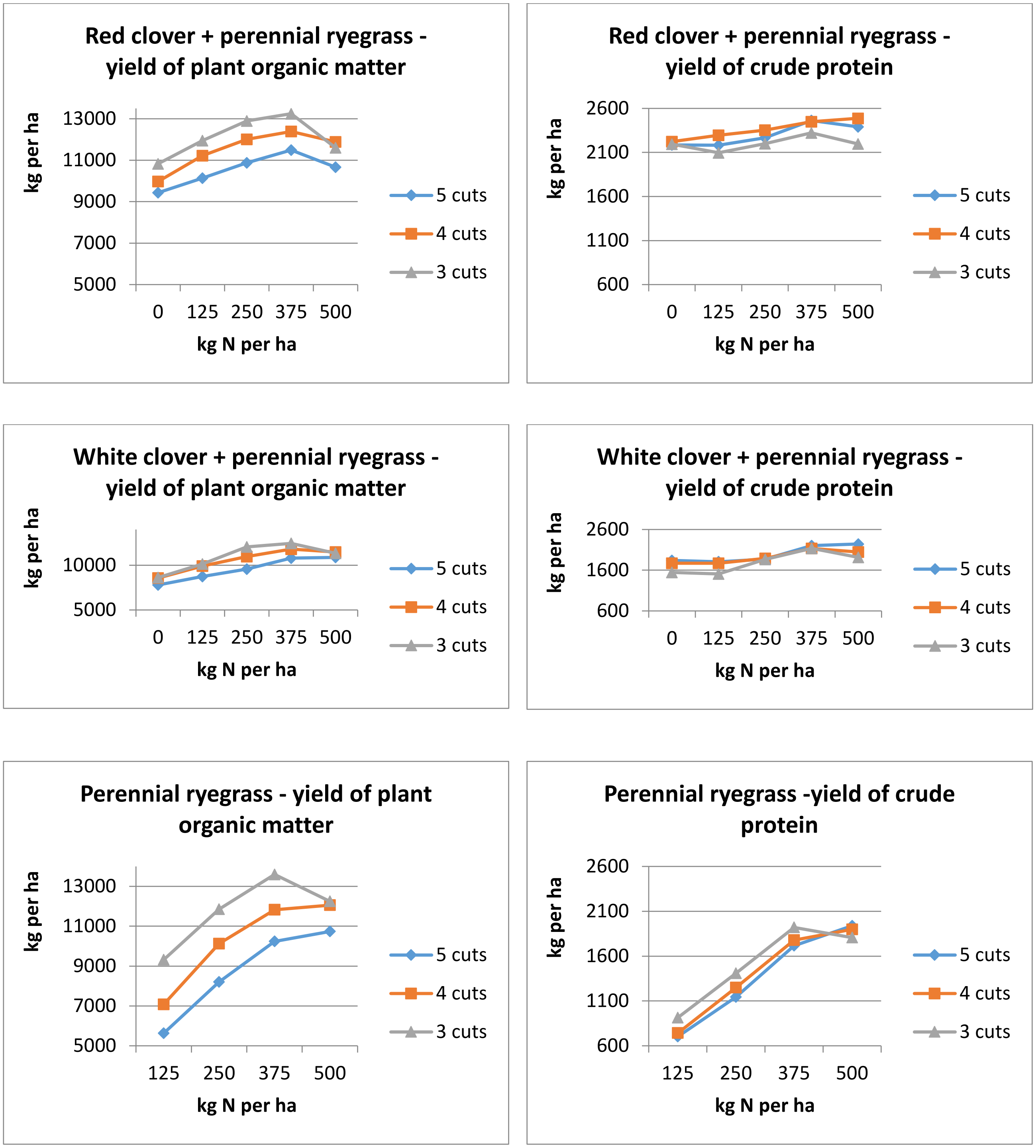


Figure 2.2. Yield of biomass and protein in a red grass-white clover mixture and perennial ryegrass depending on N fertilization and number of cuts (After Pedersen and Møller, 1976).

Sørensen and Grevsen (2015) investigated the influence of number of cuts in unfertilized crops of red grass-clover mix and white clover on total biomass and N yield over the season. Four cuts compared to two cuts per year resulted in a slightly higher N yield and a lower C:N ratio in the harvested biomass. Thus, the C:N ration in red clover and clover-grass was reduced from 17 to 13 with four compared to three cuts. In white clover, the changes were smaller.

Ryegrass for grazing and biorefining can be classified as early, medium and late heading varieties due to their phenological traits. Yield and quality of grass varieties and mixtures for forage are tested in the national yield trials for grass for forage conducted by TystofteFonden and SEGES. In comparison of a 2nd year cutting of an early, medium and late heading mixtures of ryegrass varieties only minor variation in yield as well as the composition of crude protein, sugar, NDF, crude fibre and crude ash is observed demonstrating that earliness of varieties is not important when it comes to quality for biorefining (www.sortinfo.dk) (Figure 2.3).

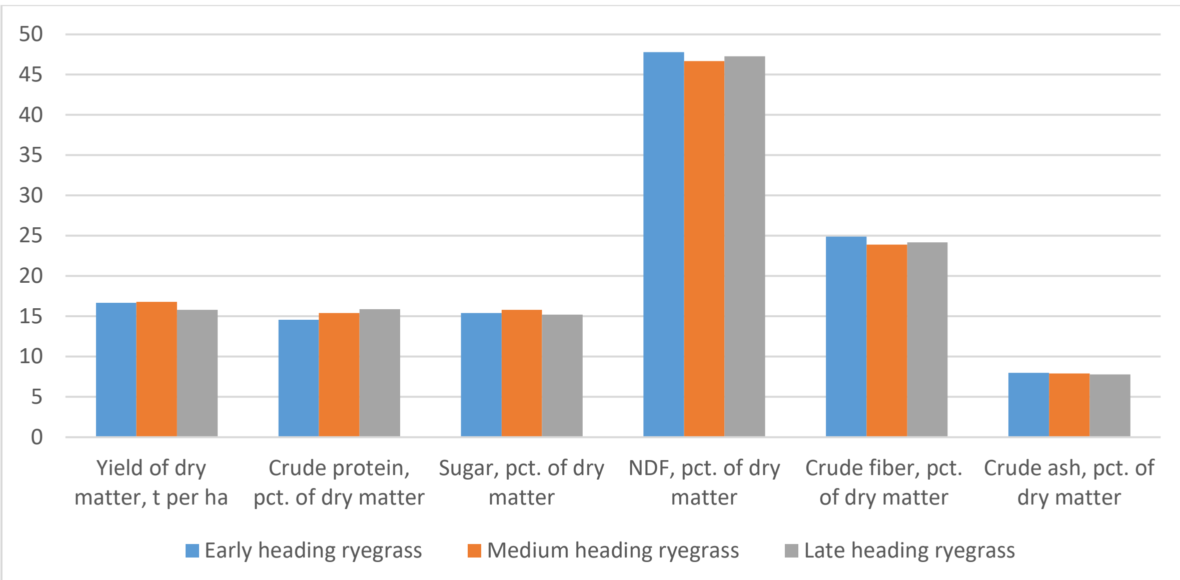


Figure 2.3. DM composition of early, mean and late heading mixtures of ryegrass (National trials, grass for forage, 2nd year cutting, 2020, www.sortinfo.dk).

The yield of mixtures of ryegrass are higher in the 1st and 2nd year of cutting than in the 3rd year of cutting with no major differences in composition of the biomass. Thus, the variation in the content of crude protein, sugar, NDF, crude fibre and crude ash between 1st, 2nd and 3rd year cutting (Figure 2.4) are limited as shown in the national yield trials for grass for forage (www.sortinfo.dk). The quality of the harvested biomass for biorefining is equivalent.

The changes in chemical composition as illustrated above are important to take into account when deciding the production strategy for green biomass and considering what it is aimed for in the biorefinery process.

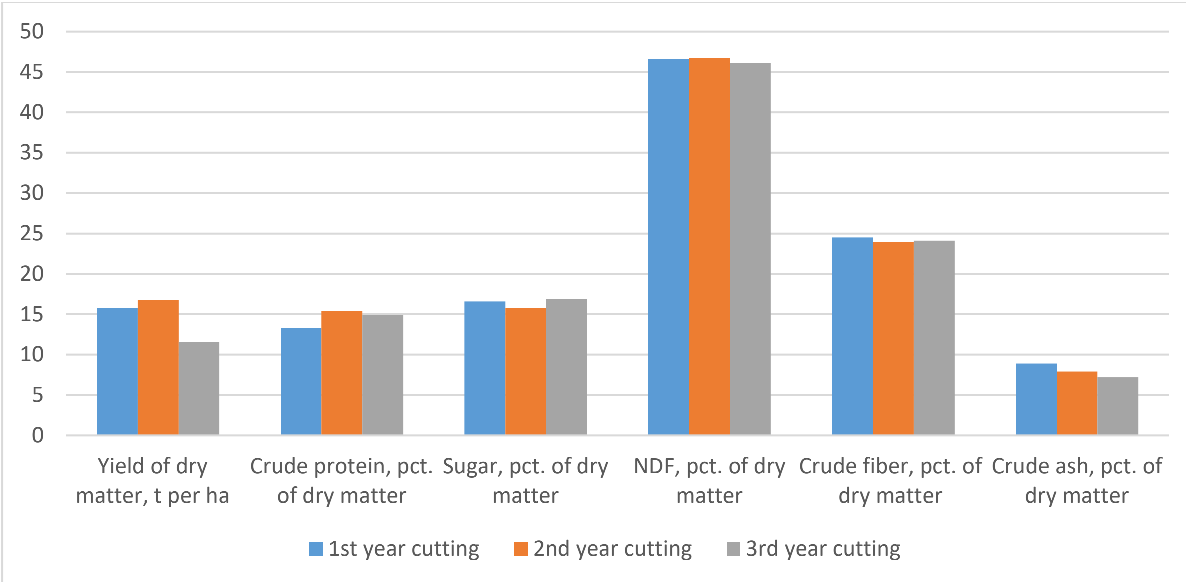


Figure 2.1. Yield and DM composition of medium heading mixtures of ryegrass (National trails, grass for forage, 2020, www.sortinfo.dk)

When the focus is on achieving high value protein for food and feed protein from green biomass, the fraction of soluble and precipitable protein is the most important constituent. The influence of the production strategy on this fraction is not completely understood. However, Solati at al. (2017) showed that there was a significant decline in crude protein content of the legumes white clover, red clover and alfalfa and perennial ryegrass and tall fescue grasses across the spring growth, where total protein changed from 30 to 15% of DM. A larger decline in crude protein with increasing maturity was observed for grass species compared with legumes. Red clover showed a significantly lower proportion of soluble true protein than did white clover. As appears from Figure 2.2 - and which is confirmed by Thers et al. (2021) – total protein yield per ha is typically higher in red clover and white clover than in moderately fertilized perennial ryegrass, but from a protein extraction point of view this may be counteracted by the lower solubility.

The work of Pedersen and Møller (1976) presented previously, showed that the true protein fraction of total N also did not change much depending on fertilization and cutting strategy, though fewer cuts and a high N-fertilization tended to reduce the proportion of true protein to total N (2-4% units).

The aspect of protein characteristics has been investigated by Thers et al. (2021). They compared and evaluated the protein quality in five forage species - white clover, red clover, alfalfa, perennial ryegrass, and tall fescue in order to identify suitable biomass for biorefining, by the Cornell Net Carbohydrate and Protein System (CNCPS). The biomass was processed and the pulp fraction and the precipitated protein concentrate analysed (Table 2.1). The DM contents of the plant material ranged from 12.6 to 20.5% and CP content from 145 to 217 g/kg across the five species. The DM content of the pulp fractions ranged from 28.0 to 42.7% and CP content from 92 to 164 g/kg DM. For the protein concentrate, the DM contents were from

15.3 to 18.3% and CP content from 266 to 336 g kg/DM. Total crude protein content in concentrate was highest for the legumes, which points to an advantage of these species in protein extraction setups. Whereas a large proportion of soluble protein for the grasses ended up in the fibrous pulp.

Table 2.1. DM and crude protein content in the five forage species; standard error in parenthesis (n = 8). Average of four harvest dates (Thers et al., 2021).

Species	Product	DM (%)	Crude protein (g/kg DM)
White clover	plant	12.7 (0.7)	217 (20)
	pulp	28.0 (1.5)	164 (15)
	concentrate	18.3 (1.4)	280 (22)
Red clover	plant	12.6 (1.0)	206 (14)
	pulp	30.7 (2.3)	134 (17)
	concentrate	16.5 (1.0)	297 (10)
Alfalfa	plant	16.2 (1.0)	216 (12)
	pulp	32.9 (2.4)	129 (11)
	concentrate	17.7 (0.8)	336 (11)
Perennial ryegrass	plant	16.7 (1.1)	165 (16)
	pulp	38.3 (1.9)	110 (11)
	concentrate	15.3 (1.0)	266 (5)
Tall fescue	plant	20.5 (0.9)	145 (11)
	pulp	42.7 (1.7)	92 (6)
	concentrate	16.5 (1.2)	291 (18)

The optimal composition for precipitated protein and pulp depends on several factors including plant material processed and processing efficiency and still needs final optimization, but roughly, the precipitated protein concentrate contains 40-50% protein and around 40% carbohydrates of which the majority belongs to fibre carbohydrates. Likewise, the composition of the pulp depends on the same factors and the chemical composition of this fraction is even more dependent on the composition of the starting material as variations in protein and fibre content is highly expressed in the pulp. Thus, low protein and/or fibre in the starting material give low protein and/or fibre in the pulp and vice versa. In the precipitated protein concentrate variations in starting materials is more reflected in the general yield of the fraction.

However, for feed purposes not just the amount of protein is relevant: pigs have specific requirements for the amino acids, lysine, cysteine and methionine, whereas poultry has a high requirement for the sulphur-containing amino acids, methionine and cysteine. Stødkilde et al. (2019) have shown that extracted protein concentrate from grass, clover, and alfalfa have a favourable content of lysine and methionine, but a lower content of cysteine. The higher content of methionine compensates – in a nutritional perspective – for the lower content of cysteine. Thus, the protein concentrate can, as regards amino-acid composition, substitute soy bean meal for broilers and laying hens (Table 2.2) providing a potential advantage of grass derived protein over soy. This has a big advantage in organic production systems where the use of synthetic amino acids is prohibited, and today's widespread use of conventional potato protein concentrate is under pressure due to the coming requirement for 100% organic feeding. In this production system there is a huge undersupply of protein feeds with a high content of especially methionine and lysine (around 50% within EU) and only few organic produced protein feeds can meet the required composition (Früh et al., 2014). In this context grass and forage-based protein concentrate has the possibility to fulfil this gap.

Table 2.1. Cysteine, lysine, methionine and threonine composition of plant and pulp and protein concentrate (g/16 g nitrogen) used for rats digestibility trial (Stødkilde et al., 2019)

	White clover			**Red clover**			**Alfalfa**			**Perennial ryegrass**			**Soya bean meal**
	Plant	Pulp	Protein	Plant	Pulp	Protein	Plant	Pulp	Protein	Plant	Pulp	Protein	
Cysteine	0.80	0.76	0.79	0.87	0.74	0.80	1.20	1.00	1.10	0.97	0.97	0.90	1.55
Lysine	5.27	6.21	6.26	5.82	6.27	6.67	5.91	6.01	6.62	5.37	5.64	5.55	6.29
Methionine	1.60	1.77	1.83	1.63	1.75	1.86	1.54	1.58	1.94	1.75	1.99	2.09	1.37
Threonine	4.59	4.77	4.95	4.66	4.74	5.04	4.31	4.28	4.99	4.36	4.50	4.76	4.01

2.2 Grass legume crops from arable land

Since arable land is a scarce resource globally a key issue is the land required to produce the feed- stock for the bio-refining. Potentially, grass can produce more biomass than annual crops due to their longer growing season and thus higher radiation capture in green foliage. This seems to be confirmed by Pugesgaard et al. (2015) where a grass-clover produced a mean yield of 14.8 t/ha DM over 3 years, while the mean yield of winter wheat (grain + straw) was 10.7 t/ha. Manevski et al. (2017) reached biomass yield (mean of three years following the establishing year) of 20.4 t/ha by festulolium, followed by tall fescue by 18.5 t/ha. In comparison, the biomass yield of traditional annual crops systems varied between 11 and 18 t/ha, with continuous maize being the most productive. The higher interception of photosynthetically active radiation (iPAR) in grasses than in annual crops is shown in Figure 2.5 above the aboveground biomass yield.

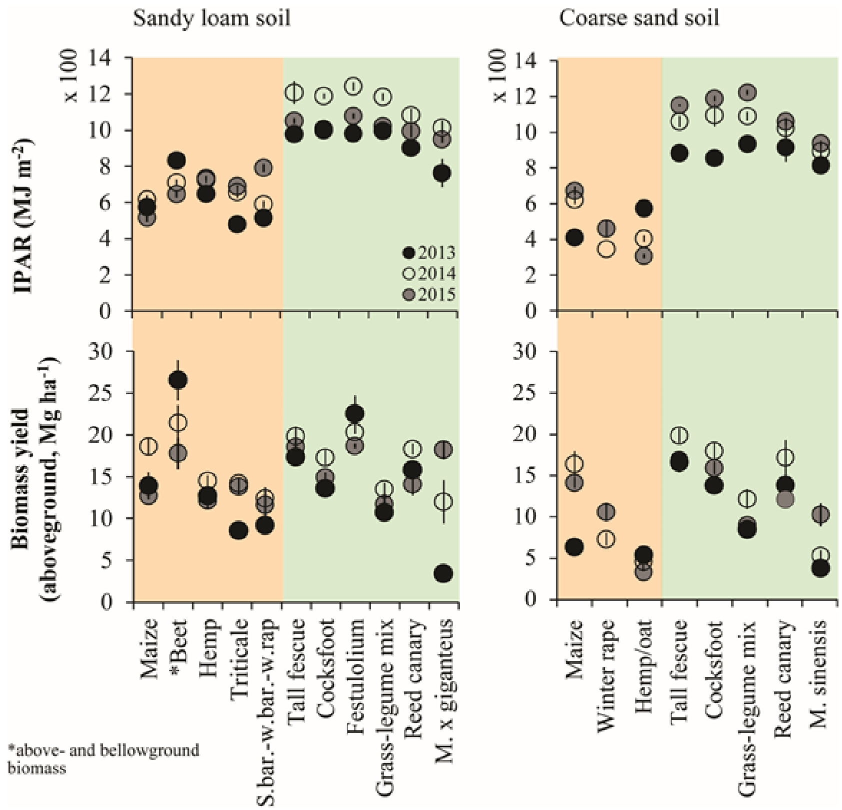


Figure 2.2. Interception of photosynthetically active radiation (IPAR) in annual (orange shade) and perennial (green shade) crops during 2013-2015 on two soil types at AU (from Manevski et al., 2017).

However, in practical agriculture grass crops are not always more productive than annual crops, which has a number of causes. Some reasons may be changed if grasses are to be used for biorefinery instead of direct animal feeding, while others may be difficult to change. In the following an overview of current yield correlations in agriculture is given.

Estimates of yield levels in Denmark of grass-clover (mixture 45 consisting of ryegrass, red clover, white clover and festulolium) and pure grass (ryegrass) are given in Table 2.3. These estimates are based on data from trials that are adjusted to yield levels in practice. Nitrogen response is based on recent fertilizer trials in the National Field Trials and at experimental stations (Madsen and Søegaard, 1991; Søegaard, 1994; Søegaard, 2004), and the yield level is set to norm yield at 2015 fertilization norms.

The level of yield is likely in many cases to increase in pure grass with 1-2 tonnes of DM/ha if other grass species than perennial ryegrass are produced, for example tall fescue or festulolium.

Grass yields most often decrease with number of years of age as also indicated in Table 2.3. How much yield is reduced over time is, however, very variable, and can be attributed to the species mix, weather conditions, fertilization and cutting frequency (Søegaard and Kristensen, 2015). In some cases, only very little yield reduction is seen with time (Eriksen et al., 2004). There is a need for better understanding these processes, and to develop recommendations to sustain productivity over time.

Table 2.2. DM yields of grass under a 4-cut strategy at different fertilization levels and at different ages of the grassland under practical farm conditions. Numbers represent net yield, i.e. net DM removed from the field (Olesen et al., 2016).

	Fertilisation (kg N/ha)	**Yield 1st-2nd year (t DM/ha)**	**Yield 3rd-8th year (t DM/ha)**
Grass-clover (mix DLF 45)	0	8.9	6.9
	240	11.5	9.5
Grass (ryegrass)	150	9.1	7.1
	300	11.1	9.1
	450	12.5	10.5
	575	13.0	11.0

All studies presented in Table 2.3 were conducted in plots where there was no tractor involved, but in practical grass-clover production at farms much traffic takes place through the season. Søegaard and Kristensen (2015) estimated a yield reduction of 1.2 t DM/ha due to the traffic on farm grassland. Recent recommendations from the agricultural advisory service are therefore to try to run the traffic in grass fields on fixed trails. The effect of traffic on the annual decline of net grass yield has not been studied.

The grass-clover in the example in Table 3 is chosen to be DLF mixture 45, which is a most used highly productive mixture, and it includes both white and red clover. Red clover is not permanent, so the lower producing white clover will take over after a few years. This in itself will reduce the yield as white clover and grasses cannot compensate for the high red clover productivity. There is no basis for a more detailed estimation of yield decline over time. We have set it to be 0.7 t DM/ha for each year after the second year of use.

Likewise, it is difficult to obtain good data on yield of forage crops in practical farming. Kristensen (2015) compared the realized yield at cattle farms of grass-clover crops and maize with the standard yield used for environmental planning. While there was a good agreement for grass-clover grass (realized yield approx. 400 kg DM/ha lower than standard), for maize the realized yield was approx. 1,600 kg DM lower per ha than standard.

Except for white-clover and mixed crops containing white-clover the DM yield per ha typically decreases with the number of cuts (Figure 2.6). This is particularly the case with tall fescue showing the highest yield of the investigated species. However, at the same time the feed quality increases, which several studies have documented within the range of 3-7 cuts per year. Tests have shown that the optimal number of cuttings to produce a high quality feed for dairy cattle is five for mixtures containing red clover and festulolium or tall fescue, and four for mixtures that do not contain the aforementioned species (Søegaard and Kristensen, 2015).

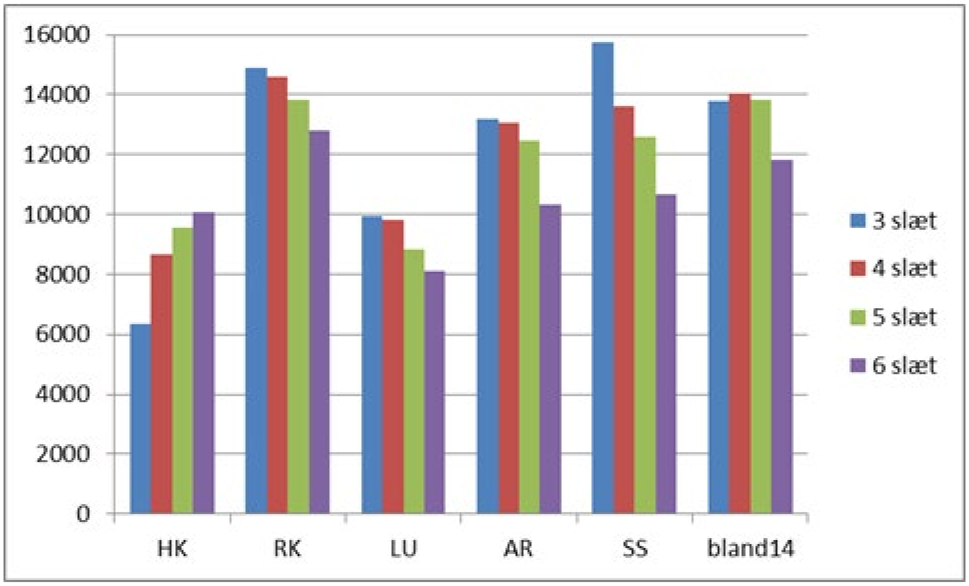


Figure 2.6. DM yields (kg/ha) of grass and clover species with cut strategies from 3 to 6 cuts (slæt) per season. HK: white clover, RK: red clover, LU: alfalfa, AR: perennial ryegrass, SS: festulolium, bland14: grass clover (mix DLF 45). Preliminary results from ongoing results at AU-Foulum (Søegaard, unpublished).

Knowledge of the variation of extractable protein amount in legumes and grasses as affected by harvest time is important for identifying optimal combinations to enable a high protein production in a biorefinery as well as the total DM yield. Research at Aarhus University have investigated the quality of protein with regard to its availability to animals using the Cornell Net Carbohydrate and Protein System (CNCPS) (Solati et al., 2017; Thers et al., 2021). The main aspect is whether the biomass is to be used for lignicellotic biorefining or for protein refining as discussed in chapter 4. With regards to protein refining total recovery in concentrate was highest for the legumes, which points to an advantage of these species in protein extraction setups (Thers et al., 2021). Solati et al. (2017) found that the estimated extractable protein [g kg/DM (DM)) defined as the easily available protein fractions B1+B2 was significantly higher in white clover and alfalfa at all harvests while, if the more cell wall attached protein fraction B3 can be extracted, white clover had the highest extractable protein amongst all species (Figure 2.7).

Future studies should look more into cut dates and management, e.g., fertilization, and how this influences the distribution between the net carbohydrate and protein fractions. However, this need coupling with estimates on best performance set-up of bio-refinery concepts in order to be able to prepare full chain evaluations of optimal combinations.

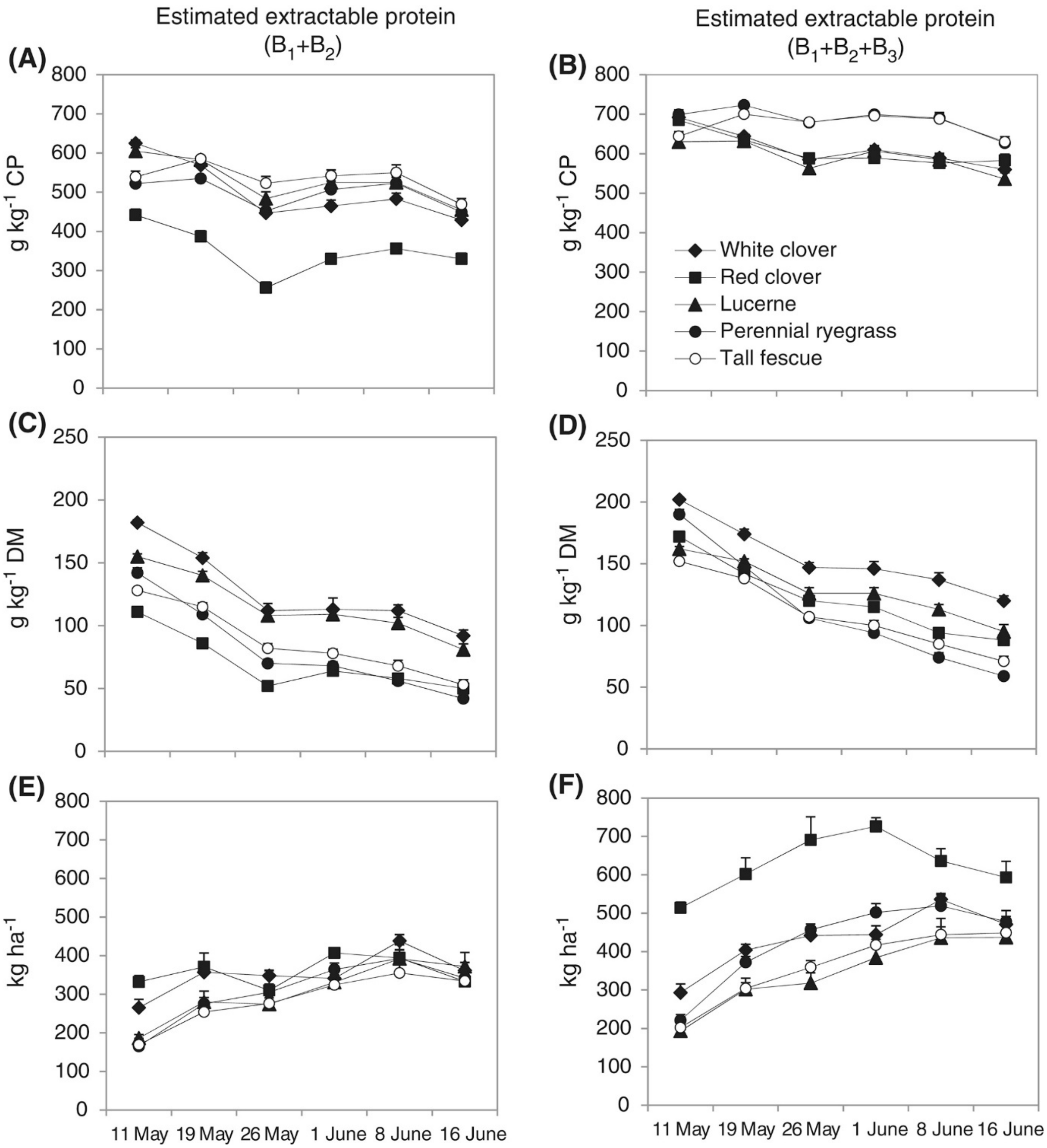


Figure 2.3. Estimated extractable protein defined as B1 + B2 (left side shown with letters A, C, E) and B1 + B2 + B3 (right side shown with letters B, D, F) in legume and grass species across the harvests during the spring growth. Data represent least square means and standard error (Solati et al., 2017).

2.3 The potential of cover crops

While the growing of grass or clover as a main crop on arable fields competes with other types of production, an alternative option to produce green biomass is to use cover crops in-between the cereal crops during the autumn period. The inclusion of unfertilized cover crops in the crop rotation is mandatory in specific areas as a mean to reduce nitrate leaching. When used for this purpose, the term “catch crop” is sometimes used. In Denmark, cover crops are currently used on approx. 500.000 ha (Landbrugsstyrelsen, 2021). Cover crops could be considered as a resource for biorefining, provided that enough biomass is produced to make the harvest profitable. However, this may not be the case, with the current average biomass production being approximately 1 tonne of DM per ha across different cover crop types, based on data from agricultural fields in Denmark (SEGES, 2020).

Nonetheless, as shown by De Notaris et al. (2019), there is a potential for optimizing cover crop growth, making it possible to turn cover crop production into a business opportunity rather than just a legal obligation. This holds several perspectives:

- Farmers might be more focused on good cover crop establishment if the crop is to be harvested and used, resulting in a better function of the cover crop in relation to reduction of N leaching risks
- Total productivity of Danish agriculture will be increased, as today the cover crops are an unused biomass resource, albeit it has a nutrient value for the subsequent crop in the crop rotation
- New research indicates that retaining cover crop residues in the field releases significant amounts of the potent greenhouse gas nitrous oxide. Harvesting the top will likely reduce this problem.

Legume species, such as Persian clover, kidney vetch, red clover and black medic, have been shown to produce a greater biomass compared to other common cover crops, as reported by Askegaard & Eriksen (2007) (Table 2.4), even though cover crop biomass values are highly variable for both legume and non-legume species (SEGES, 2020). Due to their ability to use atmospheric N through biological N2 fixation, legumes have an advantage compared to non N2-fixing species, especially when soil N availability is limited (De Notaris et al., 2021). In addition, N content in legume cover crop tops is generally greater than in non-legumes, with an average of approximately 3% for the legume species investigated by Askegaard & Eriksen (2007) and values above 4% for vetch (Buchi et al., 2015; De Notaris et al., 2021).

Several studies have shown how using cover crop mixtures including legume non-legume species optimized the provision of ecosystem services, due to increased functional diversity (e.g., Tribouillois et al., 2016). As reported by Mortensen et al. (2021), the inclusion of legumes in cover crop mixtures does not compromise the biomass yield of non-legumes, on the contrary it adds to the total cover crop biomass yield. In cases with high soil fertility non-legumes compete well, and in cases with low initial soil fertility the proportion of legume biomass increases, thus stabilizing the total biomass yield (Mortensen et al., 2021). Using cover crop mixtures including both legumes and non-legumes allows the plant system to regulate N2-fixation to its demand, resulting in higher biomass yield but without increased risk of N leaching (Sørensen et al., 2020; De Notaris et al., 2021).

When N availability is a limiting factor for cover crop growth, another option is to fertilize the cover crop in order to increase DM yield. If cover crops with improved productivity are harvested and removed, their fertilization is unlikely to increase nitrate leaching. A short-term study indicated that even if the cover crop is fertilized a reduction in nitrate leaching may be achieved if the main crop is harvested early, prolonging the cover crop growing season by 3 weeks (Jensen, 2016). Similarly, De Waele et al. (2020) found that fertilizing cover crops with a small dose of N increased cover crop biomass but not nitrate leaching, provided that cover crops were sown before the last week of August.

Adjusting the agronomic management of the main crop is another option to optimize cover crop growth. Cover crop biomass is linearly correlated with the cumulated growing degree days (temperature sum) from harvest of the main crop to early November (De Notaris et al., 2018). Thus, early sowing of the cover crop would be a key strategy to increase cover crop biomass. This can be achieved by undersowing the cover crop in early May, provided that the competition with the main crop is avoided (De Notaris et al., 2019), and/or by early harvest of the main crop (Pullens et al., 2021). Undersowing of the cover crop in May requires that the main crop is sown at a larger row distance than the usual 12 cm for grain cereals (e.g., 24 cm), to avoid competition for light and other resources (De Notaris et al., 2019). However, the relative increase in cover crop aboveground biomass reported by De Notaris et al. (2019) was mostly relevant when cover crop biomass was poor to begin with. Thus, the quantitative increase in cover crop biomass was not high enough for a profitable harvest. Conversely, a pronounced increase in cover crop biomass could be achieved by harvesting the main crop at physiological maturity (Pullens et al., 2021), which is earlier than normally done. In their study, Pullens et al. (2021) showed that, based on the linear correlation between cover crop biomass and temperature sum, harvesting winter wheat and spring barley at their physiological maturity in Denmark would allow reaching a cover crop aboveground biomass > 4 t/ha.

Earlier harvest of the main crop will require gas-tight storage of grain because the water content in the main crop is higher than at normal harvesting time. Additionally, it may be advantageous to apply strip harvest for the early harvest. By this method ears and kernels are stripped from the straw (Madsen, 2000), which can then be harvested shortly after or later (Jørgensen et al. 2013). This will reduce harvesting costs and can provide a better feed quality (Poulsen, 2010). Strip harvesting is less dependent on the weather, and total yield of digestible matter is usually larger than if the grain is harvested at full maturity with combine harvester.

Table 2.4. Aboveground DM, total N and N content as well as apparent N_2 fixation in the catch-crop species measured at the beginning of November, the corresponding N_{min} of the 0–100 cm soil layer, and the nitrate-N share of total N_{min} (average of years) (Askegaard & Eriksen, 2007).

Cover crop	Type	DM (T / ha)	Total N (kg / ha)	N content (% of DM)	N_2 fixation (kg / ha)	Soil N_{min} (kg / ha)	Soil Nitrate-N (% of Nmin)
No cover crop						24	66
Persian clover	Legumes	2.7	64	2.4	52	25	44
Kidney vetch		2.6	67	2.6	56	16	29
Red clover		2.3	61	2.7	50	20	39
Black medic		2.0	61	3.1	49	16	29
White clover		1.8	55	3.1	44	22	32
Lupine		1.2	33	2.8	21	18	41
Rye/hairy vetch		1.0	39	3.9	28	19	37
Chicory	Non-legumes	0.8	12	1.5		10	25
Ryegrass		0.6	13	2.2		13	31
Sorrel		0.5	10	2.0		12	28
Fodder radish		0.4	11	2.8		12	28
$LSD_{0.05}$		0.7	19	-	22	n.s.	13

Li et al. (2015) tested the effects of harvesting cover crops late October compared with the usual practice (without harvest) in an organic cropping system. The N recovery in the following spring barley varied significantly with type of cover crop (leguminous or not) and depending on harvest. The legume-based cover crops showed a potential to increase yield of the following main crop, but this effect was reduced with harvesting of the cover crop biomass. Such effects of modified cover crop strategies will need to be implemented in the N-regulation where currently a general residual N-effect of cover crops in the following crop is given, independently on inclusion of legumes in the mixture and harvesting.

So far, the focus of using cover crops has mainly been on their use for biogas. If protein extraction is to be pursued, more knowledge of content and extractability across cover crop species and management options must be achieved. A particular concern could be the presence of straw residues from the main crop that might impact on the juice extraction and the quality of the pulp.

2.4 Biomass from peatland and lowland areas

6.5% of the Danish agricultural area are located on organic soils, of which 98,000 ha are lowland soils with 6 - 12% organic carbon, and 73,000 ha are peatlands with >12% organic carbon (Greve et al., 2019).

Maintaining drainage on these areas for traditional agriculture will be increasingly challenging due to higher climate-induced precipitation rates as well as the need to reduce agricultural greenhouse gas emissions. However, with subsidization of rewetting, which reduces organic matter breakdown, and conversion to permanent grassland supported by the Danish Environmental Protection Agency, there is an increasing opportunity for biomass supply for biorefining from organic soils.

2.4.1 Yield of fertilized permanent grassland on organic soils

The attainable yield of permanent grassland on organic soils depends on type of species and cultivars, sward age, annual harvest frequency, and fertilization rates. On well-drained areas, fertilized permanent grassland is for several years after establishment expected to produce the same yield as grass in rotation. However, if not well-drained, the typical DM production is estimated to between 70 and 80% of grass in rotation (Nielsen, 2012). However, cultivation of flood-tolerant species, e.g. reed canary grass, festulolium and tall fescue on wet or temporarily flooded organic soils, also known as paludiculture, has documented with high annual yields up to 10-19 t DM/ha (Kandel et al., 2013; Kandel et al., 2016; Nielsen et al., 2021a, Nielsen et al., in preparation). This is comparable to productivity of grass in rotation on drained soils under similar fertilization rates of 160 – 240 kg N/ha per year. However, climatic factors might lead to annual variations in yield.

In relation to biorefining, the relevant protein content in grass biomass depends on nitrogen availability, frequency and timing of cutting, and hence plant maturity. Recent research found crude protein contents of up to 2.9 – 3.4 t/ha/year, and with biorefining techniques precipitated protein concentrates of up to 1.2 – 2.2 t/ha/year, for reed canary grass and tall fescue, cultivated on wet organic soils (Table 2.5; Nielsen et al., 2021a). Nonetheless, optimal timing of harvest remains as the most critical factor for biomass and protein yields.

Table 2.5. Total annual average biomass yields in DM, averaged crude protein (CP) contents, and averaged yields of precipitated protein concentrate per harvest and treatment for reed canary grass and tall fescue. All yields in t/ha/year. Standard deviation (SD) is given in brackets. Missing SD values due to only one replicate as a consequence of insufficient biomass for processing. NA's due to a lack of sufficient biomass for processing with the screw-press (Nielsen et al., 2021a).

Annual cuts	Reed Canary Grass			Tall Fescue		
	Yield	CP	Protein concentrate	Yield	CP	Protein concentrate
Two Cuts						
1	8.8 (± 3.3)	2.0 (± 0.7)	0.8 (± 0.2)	7.9 (± 1.9)	1.8 (± 0.5)	0.7 (± 0.2)
2	6.8 (± 4.4)	1.4 (± 0.9)	0.9 (± 0.4)	5.5 (± 2.9)	1.1 (± 0.4)	0.5 (± 0.3)
Annual sum	**15.6 (± 7.7)**	**3.4 (± 1.6)**	**1.7 (± 0.6)**	**13.4 (± 4.8)**	**2.9 (± 0.9)**	**1.2 (± 0.5)**
Three Cuts						
1	1.6 (± 0.7)	0.3 (± 0.2)	0.3 (± 0.1)	1.3 (± 0.8)	0.3 (± 0.2)	0.2 (± 0.1)
2	5.5 (± 1.4)	0.8 (± >0.0)	0.4 (± 0.1)	5.1 (± 2.7)	0.8 (± 0.3)	0.4 (± 0.2)
3	3.2 (± 0.7)	0.8 (± 0.2)	0.5 (± 0.1)	2.6 (± 1.2)	0.7 (± 0.3)	0.4 (± 0.3)
Annual sum	**10.3 (± 2.8)**	**1.9 (± 0.4)**	**1.2 (± 0.3)**	**9.0 (± 4.7)**	**1.8 (± 0.8)**	**1.0 (± 0.6)**
Four Cuts						
1	2.0 (± 0.7)	0.4 (± 0.1)	0.3 (± 0.1)	1.0 (± 0.7)	0.2 (± 0.1)	0.2 (± 0.1)
2	4.0 (± 1.3)	1.1 (± 0.3)	0.5 (± 0.1)	2.7 (± 1.6)	0.7 (± 0.4)	0.3 (± 0.2)
3	8.8 (± 4.5)	1.5 (± 0.6)	1.1 (± 0.5)	4.7 (± 0.3)	1.1 (± 0.2)	0.6 (± 0.2)
4	0.6 (± 0.5)	0.3 (± NA)	0.3 (± NA)	1.9 (± 3.4)	0.3 (± NA)	NA
Annual sum	**15.4 (± 7.0)**	**3.3 (± 1.0)**	**2.2 (± 0.7)**	**10.3 (± 6.0)**	**2.3 (± 0.7)**	**1.1 (± 0.5)**
Five Cuts						
1	1.7 (± 0.6)	0.3 (± 0.1)	0.3 (± 0.1)	0.9 (± 0.5)	0.2 (± 0.1)	0.1 (± 0.1)
2	3.9 (± 1.0)	1.1 (± 0.2)	0.4 (± 0.1)	2.4 (± 1.8)	0.6 (± 0.4)	0.3 (± 0.2)
3	6.5 (± 1.3)	1.1 (± 0.2)	0.4 (± 0.1)	4.7 (± 1.2)	0.8 (± 0.2)	0.4 (± 0.1)
4	2.4 (± 2.1)	0.6 (± 0.6)	0.4 (± 0.3)	1.1 (± 0.1)	0.3 (± >0.0)	0.1 (± 0.1)
5	0.4 (± 0.1)	NA	NA	0.7 (± 0.5)	0.3 (± >0.0)	0.3 (± >0.0)
Annual sum	**14.9 (± 5.1)**	**3.1 (± 1.1)**	**1.5 (± 0.6)**	**9.8 (± 4.1)**	**2.2 (± 0.7)**	**1.2 (± 0.5)**

2.4.2 Yield of permanent grassland on organic soils without fertilization

If the grass sward is not fertilized, only a very moderate DM yield of 2-4 t/ha/year can be expected after a few years of harvest (Nielsen, 2012). In addition, grass from unfertilized meadow has normally low nitrogen and protein concentrations and is therefore not suitable for protein extraction. Alternatively, the use of the grass biomass for biogas production has been proposed. However, Dubgaard et al. (2012) found in a study on biogas production, that there is no immediate financial incentive to produce grass biomass for biogas production on unfertilized permanent grassland due to harvesting costs that exceed biomass prices.

In conclusion, grass from unfertilized permanent grassland may represent an opportunity if focus is on the fibre part of the grass. However, if focus is also on the protein part, it is required that the permanent grass is fertilized with nitrogen, which in some cases may counteract other environmental issues as well as national and international frameworks and directives.

2.5 Harvesting and storage on wet organic soils

Even though the transition to harvest on wet organic soils might depict an initial hurdle, efficient and functional equipment for harvest on wet organic soils has been developed in recent years. The easiest transition to harvest on wet organic soils is to use adapted standard machinery. However, a soil pressure of maximum 100 g cm^{-2} needs to be met, and shear and tension forces minimized (Schröder et al., 2015). For minor areas, small machinery (e.g. https://www.agria.de/en-gb/) might be a cheap, but intensive, option. To avoid sod destruction on soft ground conditions in connection with ground pressure by machinery, crawler type vehicles for biomass harvest on challenging ground conditions have been developed by e.g. Hanze Wetlands (http://www.hanzewetlands.com/en), De Vries Cornjum, (https://www.devriescornjum.nl/en) and Loglogic (https://www.loglogic.co.uk/). This development is most promising for biomass production on wet organic soils, allowing bigger machines with more power and various technical options. However, good logistical planning and the choice of whether a one-, two-, or three-stage harvest shall be applied, is crucial.

2.6 Improvement potential by new varieties

The key to the creation of new crop varieties with improved protein production for biorefining lies in the systematic exploration of genetic variation and the selection of new phenotypes. Genetic variation is the foundation for plant breeding by providing genetic resources to accumulate favourable alleles or genes that are linked with target traits. There are two approaches that broaden genetic variation for plant breeding; natural genetic variation and creating genetic variation that does not exist in the target crops using CRISPR/Cas9. Several targets can be modified simultaneously using CRISPR/cas9, enabling pyramiding of multiple traits into an elite background. Regulatory elements can e.g. be targeted enabling targeted trait improvement. Furthermore, targeting domestication genes using CRISPR/Cas9 allows for wild- and semi-domesticated species to be domesticated and used as crops.

Traditional plant breeding relies on phenotypic selection for identifying individuals with the highest breeding value, but phenotypic selection has made little progress for complex traits such as protein yield and composition due to challenges in measuring phenotypes. Genomic selection (GS) introduced in 2001 by Meuwissen et al. (2001) presents a new alternative to traditional plant breeding that has the potential to improve selection gain in a breeding program. GS can improve breeding progress through increased selection intensity and decreased cycle time, thus accelerating gain from selection (Heffner et al., 2009, Bernardo, 2010). In GS, genome-wide DNA markers are used to predict the best performing breeding material for variety development (Meuwissen et al., 2001). These genomic estimated breeding values (GEBVs) are output from a model of the relationship between the genome-wide markers and phenotypes of the individuals undergoing selection (Figure 2.8).

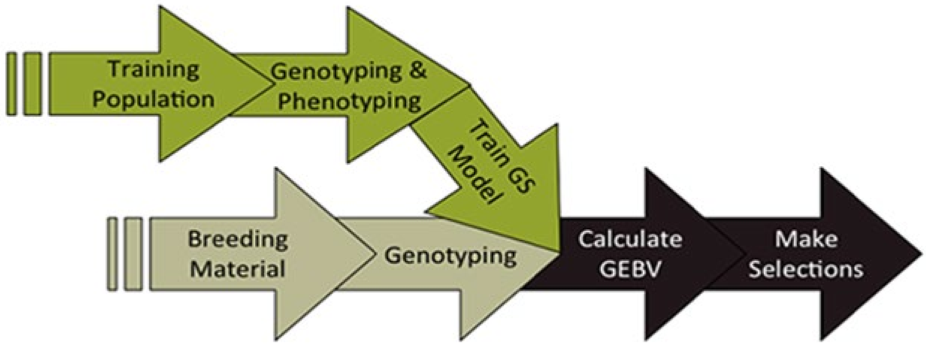


Figure 2.8. Outline of the Genomic selection processes (Heffner et al., 2009).

GS have in recent years successfully been implemented in the breeding programs of all major crops in Denmark as part of public-private partnership projects between breeding companies and universities, including crop species of relevance for protein production for bio refining such as perennial ryegrass (*Lolium perenne*) (Fè et al., 2016) and lucerne (*Medicago sativa*) (Jia et al., 2018).

With advances in GS, data volumes and complexity have increased dramatically, leading to novel interdisciplinary research efforts to integrate computer science, Artificial Intelligence, quantitative genetics, and bioinformatics in plant breeding (Harfouche et al., 2019). Developing and applying these interdisciplinary research efforts can potentially further accelerate breeding for protein production for biorefining with improved yield potential and stability. In general, a future opportunity for Artificial Intelligence is to support decision-making processes in agriculture.

3 Environmental impacts related to crop production

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3.1 Grass and legumes in rotation

3.1.1 Leaching of nitrate

Pure cutgrass under unfertilized conditions has a marginal leaching (<5 kg N/ha), and by adding up fertiliser to the economic optimum for plant growth nitrate leaching is still quite low (<20 kg N/ha) (Olesen et al., 2016). Thus, Whitehead (1995) refers a number of studies that by adding up to 500 kg N/ha/year for grass showed no leaching above the above-mentioned low level. It agrees well with the Danish studies where leaching in the 4th-5th year ryegrass with supply of 300 kg N/ha was 12-20 kg N/ha (Eriksen et al., 2004). With increasing age of the pasture there was a tendency for increased leaching and the leaching in the 6th-8th ryegrass year was on average 38 kg N/ha in the same experiment. Recent experiments with festulolium fertilised 400-500 kg N/ha/year over the first 3 years of production leached 7-21 kg N/ha on a loamy sand, while 27-74 kg N/ha was leached from cocksfoot on a coarse sand (Manevski et al., 2017). In the following years leaching increased on the loamy sand (Kiril Manevski, pers. comm., May 2021), showing that fertilization should be adjusted over time or that reestablishment may prove more efficient to keep high productivity and low nitrate leaching.

In cut grass-clover, leaching under unfertilized conditions is found to be in the range of 14-21 kg N/ha, and not differing significantly with the age of the crop (Eriksen et al., 2004, 2015; Manevski et al., 2018; Kiril Manevski, pers. comm., May 2021). Fertiliser application within the economic optimum for plant growth has only limited effect on nitrate leaching - in the range of 2-3 kg N/ha (Eriksen et al., 2015; Wachendorf et al., 2004). The more fertilizer that is applied to a grass-clover, the lower the clover content will become and nitrate leaching will approximate that of pure grass.

From the above, Table 3.1 summarises an estimated N leaching. It should be emphasized that this is an estimate, since there are no published Danish experiments with the determination of nitrate leaching by increasing fertilizer application to grass or grass-clover with current agronomic practices. However, new experiments with determination of nitrate leaching by increasing fertilizer application have been performed in grass-clover and preliminary analysis indicates that within the recommended fertilizer standard of around 300 kg N/ha, leaching is relatively low and comparable to numbers in Table 3.1 with increasing marginal leaching at higher levels (Jørgen Eriksen, pers. Comm., May 2021).

It is expected that the effects of soil type on leaching is only limited for grasses, even though the results from Manevski et al. (2018) indicate a higher leaching risk on coarse sand. The estimates in Table 3.1 are for grasses produced for feed, which are mainly perennial ryegrass. However, for biorefining it seems that high protein yields can be achieved from highly productive *Festulolium* or *Festuca* varieties (Morten Ambye-Jensen, pers. comm.) that have a higher biomass yield potential (Becker et al., 2020; Pedersen, 2012), and probably also a higher N-uptake potential.

Table 3.1. Estimated nitrogen leaching (kg N/ha/year) from cut grassland at different fertilisation and age (Olesen et al., 2016).

Pure grass (kg N/ha/y)			Grass-clover (kg N/ha/y)		
N-fertilisation	**1.-2. year**	**3.-8. year**	**N-fertilisation**	**1.-2. year**	**3.-8. year**
0	5	5	0	15	15
150	15	15	120	20	20
300	20	30	240	20	30
450	25	35			
575	55	70			

For comparison nitrogen leaching from grain and maize is shown in Table 3.2. The crops chosen to compare with are winter wheat and maize grown continuously. It is assumed that maize is grown with a cover crop, but often cover crop does not develop well in maize. The calculations in Table 3.2 are made with NLES5 (Børgesen et al., 2020). There is no data for maize in combination with cover crops in NLES5, and it is not reasonable to assume the same effect of cover crops as in a cereal crop since a cover crop in maize is not developed to the same level of N-uptake. Instead, we have anticipated in the model calculations that the cover crop in maize has a similar effect during winter as a winter cereal crop. The calculation includes the statutory pre-crop effect of cover crops of 25 kg N/ha to be subtracted from the following years N allocation.

Table 3.2. Nitrogen leaching in winter wheat and maize by economically optimal fertilization level in an area rich in animal manure (calculated by NLES5, Christen Duus Børgesen, May 2021).

Crop	Soil type	Fertilisation (kg N/ha)	Leaching (kg N/ha)
Winter wheat	Sand (irrigated)	63 + 140*	72
	Clay	90 +140*	54
Maize	Sand (irrigated)	63 +140*	99
	Clay	52 + 140*	62

* Total N with manure

Comparing Table 3.1 and Table 3.2 it is clear, that grass production in almost all circumstances causes significantly less nitrate leaching than the production of wheat and maize. However, care should be given to adjust fertilisation levels of pure grass to the level of crop removal, especially when the crop is older than 2 years.

Another issue is when the grass or grass-clover sward is ploughed after end of use or for reseeding. At this point there is a significant risk for a substantial nitrate leaching, probably in particular for grass-clover swards. Hansen et al. (2007) showed, however, that this risk could be reduced substantially if the grass-clover sward after ploughing in March was followed by an unfertilized barley crop with under-sown cover crop. Thus, the nitrate leaching was reduced by 66 – 80% compared to barley with no cover crop and an intensive tillage after harvest. The maximum reduction in nitrate leaching was obtained if the barley crop was harvested before maturity allowing the cover crop to develop better. In this case the leaching was reduced to approximately 10 kg N per ha. Therefore, in order to obtain the foreseen reduction in nitrate leaching at crop rotation or farm scales, the grassland need to be either long-term with adjusted fertilisation, and/or very efficiently followed by cover crops when ploughed. These systems need further optimisation including tests of the efficiency of reseeding without ploughing on crop productivity as well as on keeping nitrate leaching low.

If a biorefinery is established in a nitrate sensitive area it will be logical that much of the area is more or less permanently cropped with grass. An effective system may be that the grass is grown for 4-8 years, depending on how well yield reduction can be controlled. Then it is ploughed in spring, and spring barley with a ley crop of grass is established in order to enter a new grass cycle. Such a system will most likely be very efficient in keeping nitrate leaching low. However, keeping the pressure of unwanted grass species such as couch grass low without herbicides may be a challenge, and test of different systems and their impacts are necessary.

3.1.2 Nitrous oxide emission

Agriculture contributed in 2018 89% of the total Danish emissions of nitrous oxide (Nielsen et al., 2020). The emission is mainly due to the cycling of nitrogen in agricultural soil, where fertilizer, manure and crop residues are direct sources of nitrous oxide emissions, while ammonia and leached N are indirect sources to nitrous oxide. In the following assessment on what land use change means for these emissions, the latest revision of the methodology recommended by the International Panel on Climate Change (IPCC, 2006) is applied, and it is also the basis for the national inventory of greenhouse gas emissions.

Emissions of nitrous oxide in a given year are linked to the land use (crop), fertilizer type (mineral or manure), nitrogen amount and method of application (manure), with a limited number of fixed emission factors linked to the various items.

The mineralisation of crop residues is an important source of nitrous oxide, and grasses develop a larger root biomass than winter wheat and maize. With perennial grass, however, the average annual contribution from this source becomes less important since only a limited part of the roots turn over each year. For the calculation of the contribution of plant residues, data from Mikkelsen et al. (2014) is used, and the amount of nitrogen fertiliser assumed for grass in rotation and grass outside the rotation is applied, respectively for 1-2 years of grass and 3-8 years of grass production.

A change in land use from cereals or maize to grass can, depending on the fertilizer level, lead to either decreased or increased nitrous oxide emissions (Table 3.3). The increase in annual nitrate leaching with increased pasture age (Table 3.1) will give rise to a greater indirect emission of nitrous oxide, but it is offset by the less direct emissions from crop residues due to less frequent re-establishment.

A recent study indicated that the standard IPCC emission factor of 1% of applied fertiliser converted into nitrous oxide, does not hold for grasses (Baral et al., 2019). This experiment was conducted at the sandy loam site at AU Foulum and showed that grasses (festulolium and tall fescue) fertilised 425 kg N/ha and maize fertilised 140 kg N/ha gave rise to emission factors of 0.23 +/- 0.04, 0.32 +/- 0.03 and 0.54 +/- 0.13%, respectively. The values may be higher on loamy soils but the lower emission factors for grass than for an annual crop indicates that fast fertiliser uptake in growing grass can keep nitrous oxide emissions low. However, this needs considerably more documentation, especially if a derogation from the use of standard IPCC in the national emission accounting is to be applied for.

Baral et al. (2020) investigated the emission during renovation (cultivation) of a grass sward. They examined the effect of renovating a six-year-old festulolium crop on N_2O emissions. As a secondary objective, the study evaluated the potential for mitigating N_2O emissions in spring by spraying the sward with a nitrification inhibitor containing 3.4-dimethylpyrazole phosphate (DMPP) prior to cultivation. Cultivation increased N_2O emissions 2.5-fold in spring, and 2-fold in autumn, compared to uncultivated plots. Emission factors for spring barley (catch crop after cultivation), re-sown festulolium, and uncultivated festulolium during the monitoring periods were, respectively, 0.40, 0.42 and 0.12%. Spraying festulolium with DMPP delayed the transformation of ammonium to nitrate during spring. However, DMPP did not reduce N_2O emissions significantly in this study.

Table 3.3. Estimated emissions of nitrous oxide (using IPCC standard emission factors) from the cultivation of different crops at different fertilization levels measured in both nitrous oxide N and CO_2 equivalents (Based on Olesen et al., 2016).

Crop		Fertilisation (kg N/ha)	kg N_2O-N/ha/year	Tonne CO_2-eq/ha
Winter wheat	Sand (irrigated)	93 +140	2.6	1.2
	Clay	109+140	2.8	1.3
Maize	Sand (irrigated)	69+140	2.6	1.2
	Clay	44+140	2.3	1.1
Grass-clover	1-2 years	0	0.3	0.2
		240	2.8	1.3
	3-8 years	0	0.1	0.1
		240	2.6	1.2
Ryegrass	1-2 years	150	1.8	0.9
		300	3.4	1.6
		450	4.9	2.3
		575	6.3	3.0
	3-8 years	150	1.6	0.8
		300	3.2	1.5
		450	4.8	2.2
		575	6.2	2.9

Nitrous oxide emissions caused by fertilisation may be reduced by application of nitrification inhibitors. Meta-analyses have shown an average reduction of emission by 40-45% (Akiyama et al., 2010; Qiao et al., 2015). The cost of application together with fertiliser or manure is approx. 200 DKK/ha annually (Olesen et al., 2018). As this is a rather limited cost, which will have a large effect at the high levels of N-fertiliser necessary to support high protein production in high-yielding pure grasses, this can be an attractive measure to keep climate impact low even at high fertilisation and productivity. On the other hand, if grass clover mixtures or pure clover can deliver appropriate yields of total biomass and of protein with no or limited N-fertiliser, this will be the most environmentally benign production method.

3.1.3 Carbon storage

By a transition from grain cultivation to grass there will be a rapid accumulation of carbon in the soil over the first few years, after which the rate will fall and become more constant. This is because, especially in the first year, there will be a large build-up of carbon in the grass root system. Taghizadeh-Toosi and Olesen (2016) calculated an annual accumulation of carbon in the entire soil profile below productive grass around 2 t C/ha/year in the first two years after conversion, but this slowed to an annual accumulation of approximately 0.6 t C/ha/year in subsequent decades. The greater build-up of carbon in the soil in the first few years is not permanent since it mainly consists of easily degradable material. Carbon accumulation in common productive pastures can thus be set to 0.6 t C/ha/year. The annual build-up of carbon under the grass will continue over a very long period (over 100 years), and the measured carbon content in permanent grassland is typically 50 to 100% higher than for land with annual crops in rotation (Soussana et al., 2004). The above-mentioned carbon storage will probably apply to clover regardless of fertilization level, whereas carbon storage is estimated to be lower (half) at a low fertilization level in pure grass because production here is smaller and thus the supply of carbon to the soil also smaller (Table 3.4).

Little is known about the effect on soil carbon of either the composition of grassland, nor their fertilisation and cutting systems. Also, the allocation of C to roots remains largely in the dark (Taghizadeh-Toosi et al., 2020). However, Cougnon et al. (2017) found that N fertilization increased root and stubble biomass of five grass species grown in a 3-year-old ley, and that the root biomass differed between grass species, suggesting that the impact on root biomass of fertilization level and species composition of leys deserves further attention. Tall fescue showed the highest root + stubble biomass (18-19 t/ha), which points to further documentation of the potential of this species for both biorefinery and soil carbon storage.

Table 3.4. Estimates of carbon storage in grass (t C/ha/year) at different fertilization levels and at different ages of grassland (Olesen et al., 2016).

	Fertilization (kg N/ha)	**Year 1-2**	**Year 3-8**
Grass-clover (DLF mix 45)	0	0.6	0.6
	240	0.6	0.6
Pure grass (ryegrass)	150	0.3	0.3
	300	0.6	0.6
	450	0.6	0.6
	575	0.6	0.6

Earlier there has been a common understanding that tillage was an important factor in soil carbon turnover, and that its absence was one of the main causes of the higher carbon storage below perennial crops than below annual crops. Although there may still be a small effect of tillage, there is now a growing consensus that this effect is very limited, and that the annual carbon input to the soil in crop residues and animal manure is the main determining factor for the soil carbon balance. Likewise, the claimed positive effect of no-till farming on soil carbon seems rather to be a difference in carbon distribution across the soil profile than a difference in total carbon content (Powlson et al., 2014).

3.1.4 Changes in climate and environmental profile by converting from annual crops

Table 3.5 shows the calculated change (based on the former tables) in yield, N-leaching and greenhouse gas emissions on clay soils by replacing winter wheat with grass of different types and varying age under current production conditions for cattle feed. Only by cultivating pure grass with 450 kg N/ha or more, higher total yields are obtained in the grass than in winter wheat (grain and straw accumulated). In general, a reduction of N-leaching of 40-50 kg N/ha is obtained, except at the very highest levels of N-fertilization in pure grass, in which case there is no reduction in N-leaching. The reduction in greenhouse gases is about 2 t of CO_2-eq/ha but declines at the very highest level of nitrogen in the pure grass if not nitrification inhibitors are applied. Nitrous oxide emissions are less from clover and therefore the reduction in greenhouse gas emissions here are about 2 t of CO_2-eq/ha greater.

Table 3.5. Changes in annual DM yields, N-leaching and net emissions of greenhouse gases (carbon storage and nitrous oxide) by changing from winter wheat (grain + straw) to grass on clay soil (recalculated from Olesen et al., 2016).

Crop		**Fertilisation (kg N/ha)**	**Change in DM yield (t/ha)**	**Change in leaching (kg N/ha)**	**Change in GHG emission (t CO_2-eq/ha)**
Grass-clover	1-2 years	0	-2.7	-39	-3.3
		240	-0.1	-34	-2.2
	3-8 years	0	-4.7	-39	-3.4
		240	-2.1	-24	-2.3
Ryegrass	1-2 years	150	-2.5	-39	-1.5
		300	-0.5	-34	-1.9
		450	0.9	-29	-1.2
		575	1.4	1	-0.5
	3-8 years	150	-4.5	-39	-1.6
		300	-2.5	-24	-2.0
		450	-1.1	-19	-1.3
		575	-0.6	16	-0.6

Table 3.6 shows the calculated change in yield, N-leaching and greenhouse gas emissions on sandy soil by replacing whole crop maize with grass of different types and varying age under the present production conditions for cattle feed. The high yield in maize caused it in all cases to give higher yields than grass. There is a general reduction in N-leaching of 70-80 kg N/ha, except at the very highest N level in pure grass where the reduction is only half of that. The reduction in greenhouse gases is about 2 t of CO_2-eq/ha but lower at the very highest level of nitrogen in the pure grass if not nitrification inhibitors are applied. Nitrous oxide emissions are less of clover and therefore the reduction in greenhouse gas emissions here is about 2 t of CO_2-eq/ha higher.

Table 3.6. Changes in annual DM yields, N-leaching and net emissions of greenhouse gases (carbon storage and nitrous oxide) by changing from whole crop maize to grass on sandy soil (recalculated from Olesen et al., 2016).

Crop		Fertilisation (kg N/ha)	Change in DM yield (t/ha)	Change in leaching (kg N/ha)	Change in GHG emission (t CO_2-eq/ha)
Grass-clover	1-2 years	0	-4.3	-84	-3.3
		240	-1.7	-79	-2.1
	3-8 years	0	-6.3	-84	-3.4
		240	-3.7	-69	-2.2
Ryegrass	1-2 years	150	-4.1	-84	-1.5
		300	-2.1	-79	-1.8
		450	-0.7	-74	-1.1
		575	-0.2	-44	-0.5
	3-8 years	150	-6.1	-84	-1.6
		300	-4.1	-69	-1.9
		450	-2.7	-64	-1.2
		575	-2.2	-29	-0.5

It should be noted that the above calculations are with current yields of crops in practical agriculture. There seems, however, to be a higher yield difference between the most productive grasses and grain crops, which is not captured by the current management strategies in agriculture. Accordingly, experiments at AU have shown approx. twice as high yields in pure grass (festulolium) as in wheat and barley in first production years, while maintaining a reduced risk for nitrate leaching (Figure 3.1).

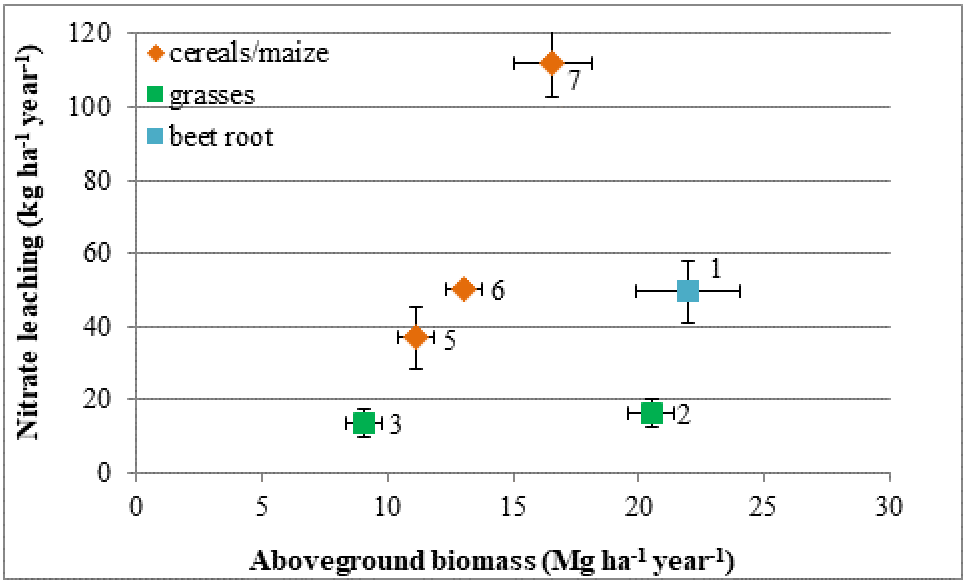

Figure 3.1. Mean nitrate leaching (based on 2013-2015) versus biomass yields for 1) beet, 2) festulolium, 3) grass-legume mix, 5) barley, 6) wheat/triticale, 7) maize, grown on the sandy loam soil at AU Foulum. Bars indicate +/- SE (Manevski et al., 2017; 2018).

3.1.5 Pesticide use

Plant protection measures for both cereals and grasses minimize yield losses in relation to weed, pest and disease management. Due to the fewer natural pests, grasses require fewer pesticides compared to cereals and maize. According to the European Environmental Agency, perennial grasses grown for industrial purpose pose rather low environmental risk in relation to pesticide pollution of soils and water, whereas maize and some grain cereals are estimated to pose a moderate-to-high level of environmental risk (EEA, 2007).

In Denmark the mean pesticide load per ha for agricultural crops was 1.42 in 2018, covering a variation from 0.01 in grass and clover to 5.16 in potatoes (Miljøstyrelsen, 2020 (table 8-4)). Oilseed rape has so far been the main energy crop in Denmark used for biodiesel production. It provides valuable protein concentrates for animal feed, and it had a pesticide load of 2.62 in 2018. Beets may be interesting for energy production due to their high productivity and protein may be extracted from the tops, but they had a pesticide load of 4.13. Grass and clover are thus the by far less pesticide treated agricultural crops today, and they can quite easily be grown organically if so wished.

3.2 Permanent grassland on organic soils

Organic soils under permanent grassland play a critical role for carbon and nutrient cycling, locally and globally. However, their environmental impact – whether positive or negative – is to the largest extent depending on soil water conditions.

3.2.1 Drained organic soils

Drainage of organic soils accelerates decomposition of organic matter, further enhancing mineralization of organic nitrogen (N) and phosphorus (P), hence leading to leaching of e.g. carbon (C), N and P into bodies of water. However, the actual effect and magnitude of leaching is strongly depending on catchment hydrology and site-specific biogeochemical conditions (Tuukanen et al., 2017).

With most organic soils being environmental sensitive areas, harvesting and removal of biomass results in an export of excess N and P from soil, mitigating nutrient leaching with efficiencies of up to 92% (Jabłońska et al., 2020). However, organic soils are very inhomogeneous, with some areas delivering a high amount of nutrients from peat mineralization and some showing a lack of potassium (K). Fertilization should hence be restricted in areas characterized by nutrient losses, while in some areas site-specific fertilization with deficit-nutrients has shown to significantly increase biomass yields as well as removal of excess N and P (Nielsen et al., 2013). In conclusion, general estimates of nutrient losses from drained organic soils cannot be made due to site-specific hydrological and biogeochemical interactions. However, biomass harvest and removal under appropriate fertilization clearly reduces the risk of nutrient leaching (Jørgensen & Schelde, 2011).

In addition, drained organic soils are hotspots of greenhouse gas emissions (GHG) due to stimulation of oxidative processes, enhancing emissions of carbon dioxide (CO_2) and nitrous oxide (N_2O).

The estimated global warming potential by GHG emissions of permanent grassland in temperate climate is reported within the range of 17 – 30 t CO_2eq/ha/year for drained fens (lowland areas), depending on drainage depth, and 25 t CO_2eq/ha/year for bog peatlands (Wilson et al, 2016). Specifically for Denmark, the range of reported GHG from agriculturally used drained organic soils is between 3.5 to 13.6 t C/ha/year and up to 61 kg N_2O-N/ha/year, respectively (Elsgaard et al., 2012, Petersen et al., 2012). Biomass production on drained organic soils does not mitigate these GHG emissions.

3.2.2 Rewetting of organic soils

Paludiculture is the term for a production system that combines rewetting and biomass production with flood-tolerant crops (Tanneberger & Wichtmann, 2011). Rewetting of formerly drained peatlands is a suggested mitigation option in terms of reducing CO_2 emissions and restoring the ecosystem carbon sink function (Joosten et al., 2012). In this context, rewetting of drained peatlands has been included as a potential target for climate change mitigation in the Kyoto protocol (IPCC, 2014). Paludiculture has further been suggested as a promising option to reduce anthropogenic CO_2 emissions from peatlands, while at the same time facilitating continued agricultural biomass production (Tanneberger & Wichtmann, 2011). In Germany, emission reductions following rewetting were reported with up to 89% for fen (lowland) organic soils, and 70% for bogs (Drösler et al., 2013). In a Danish context, it is estimated that rewetting of drained peatlands will reduce GHG emissions by approximately 3-15 t CO_2eq/ha/year (Wilson et al., 2016, Nielsen et al., 2021b). Even though rewetting of organic soils will in most circumstances lead to increases in methane

(CH_4) emissions, these will be offset by significant reductions of CO_2 and N_2O emissions (Günther et al., 2020). It is not expected that biomass harvest will have a significant negative effect on the net GHG mitigation potential of rewetting (Günther et al., 2015).

In addition to effects of rewetting and paludiculture on GHG emissions, associated effects on potential nutrient discharges to water bodies are likely. The environmental effects of a raised water table will lead to changes in leaching of nutrients as soil redox conditions are decreased due to restricted oxygen (O_2) diffusion. In this context N and P biogeochemical processes are of special interest. Anaerobic conditions favour denitrification, i.e., microbial removal of nitrate (NO_3-), possibly in competition with plant NO_{3-} uptake (e.g., Kaye and Hart, 1997). On the other hand, anaerobic conditions decrease the adsorption of P to iron (Fe) and manganese (Mn) oxides due to microbial reduction of these minerals (Hoffmann et al., 2009). Consequently, P may be released to the soil solution and discharged to downstream vulnerable recipients. Yet, the P uptake by harvested and exported crops in paludiculture may mitigate the high P mobilisation at least during the growing season (Zak et al., 2014). However, the majority of results on the prevention of nutrient leaching by paludiculture currently is known from macrophyte species such as cattail (*Typha spp.)* and common reed (*Phalaris spp.), being* not suitable as biomass feedstock for biorefineries if the focus is on protein. Hence, the effect of grass species in paludiculture on the mitigation potential of nutrient leaching remains to be more evaluated in the future.

One possibility to mitigate high nutrient discharges on organic soils is topsoil removal (e.g. Zak et al., 2017; Huth et al., 2020) prior to the cultivation of suitable perennial grasses. Topsoil removal has shown the potential to avoid elevated amounts of nutrients in topsoil, otherwise prone to leaching, while simultaneously mitigating methane emissions by a factor of up to 400 (Huth et al, 2020). However, the feasibility of such measures needs to be documented.

In conclusion, rewetting of organic soils and the subsequent conversion to permanent grassland for biomass production is a promising option to mitigate adverse environmental impacts such as GHG emissions and nutrient leaching into bodies of water, while keeping up biomass production. However, detailed site-specific magnitudes of reductions in a Danish context remain to be documented.

3.3 Environmental effects of increasing productivity and harvesting of cover crops

The area where cover crops are grown (500,000 ha in 2020) is an interesting additional biomass source in the case that cover crop growth is enough to make yield profitable. As discussed in chapter 2.3, this could be obtained by including legumes in cover crop mixtures, early fertilization and optimized crop management, such as early harvesting of the main crop. In the analyses behind "The +10 million tonnes study" (Gylling et al., 2016), and the updated scenario report by Gylling et al. (2021), it was assumed that the earlier establishment of the cover crops, combined with inclusion of legumes in the mixture, and harvesting the aboveground biomass, overall will not change the nitrate leaching compared to today's practice (Jørgensen, 2012). Some results point to the fact that leaching may even be reduced, when increased growth of cover crops is obtained by early harvest of main crop and increased N availability for the cover crop (Jensen, 2016; Sørensen et al., 2020; De Notaris et al., 2021).

Increased productivity and utilization of biomass crops would also affect the various contributions in the greenhouse gas accounts for cover crops. Nitrogen in crop residues from cover crops can contribute to nitrous oxide emissions when the residues are retained in the field (Olesen et al., 2018), offsetting or exceeding the indirect reduction in nitrous oxide emissions from reduced nitrate leaching (Table 3.7). It should be noted that such calculations are based on emission factors that are currently being revised (e.g., ResidueGas project), as the variability in soil nitrous oxide emissions is very high. In fact, results on direct nitrous oxide emissions from cover crop residues are contrasting, and depend on several factors, such as cover crop type, management, climate and soil characteristics (Abdalla et al., 2019). Harvesting cover crop aboveground biomass could reduce the risk of direct nitrous oxide emissions but this may in turn reduce soil carbon build up. Nonetheless, the contribution from the belowground cover crop carbon pool (roots and carbon deposited in the soil by the living plant) may be more important for the build-up of stable soil organic carbon (Rasse et al., 2005). If increasing the productivity of cover crops corresponds to an increase in belowground biomass and carbon deposition, the removal of aboveground biomass would not be in contrast with soil organic carbon build up, but more studies are needed to clarify this aspect.

The above complexity is exemplified by the results from Li et al. (2015), who, surprisingly, did not measure a decrease in nitrous oxide emissions after harvesting cover crops late October compared with usual spring ploughing. This may be due to increased root leakage of N and C after harvest, which supports nitrous oxide emissions. This shows that a mechanistic understanding needs to be obtained, as well as further optimization of crop management systems.

Table 3.7. Reduction in GHG emissions (kg CO_2 eq/ha/year) calculated for cover crops on sandy and clay soils at the current practice, without harvesting of biomass (Jørgensen et al., 2013; Olesen et al., 2018).

Process	**Sand**	**Clay**
Nitrous oxide from saved N-fertilisation due to reduced N-norm	94	94
Nitrous oxide from reduced ammonia evaporation (due to reduced N-norm)	1	1
Nitrous oxide from crop residues	-323	-155
Nitrous oxide from reduced nitrate leaching	115	55
Total nitrous oxide reduction	-113	-5
Soil carbon storage from cover crops	1,000	1,000
Total greenhouse gas reduction from cover crops	**774**	**990**

In general, cover crops increase biodiversity in agroecosystems where bare soil is the alternative. A further effect on biodiversity can be obtained if the cover crop mixture includes flowering species, such as clover, phacelia and fodder radish if these are allowed to reach the flowering state. If the cover crop biomass is harvested for biorefining purposes, it remains to be investigated whether an earlier harvest of the main crop can ensure both a higher biomass, and species to reach a flowering state before harvest.

4 Green Biorefining for multiple products

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4.1 Intro to green Biorefining

Green Biorefining (GB) is a fundamental concept that "represents the sustainable processing of green biomass into a spectrum of marketable products and energy" [McEniry and O'Kiely, 2014). In other words, GB is a technology platform that integrates a variety of different sustainable solutions in order to produce everything from food and feed to biomaterials, biofuels and bioenergy.

For biorefineries in general, no single end-product can typically provide economic surplus on its own (de Jong, 2009). Conversely, it is the sum of all co-process streams and products (i.e. cascade utilization) that enables the GB concept to provide both economic and environmental sustainability. Since many of the products and co-process streams can be used for different end-purposes, a successful integration of various biorefining technologies is a complex task with many possible solutions. Many factors can be considered, but central to the concept of all biorefinery systems is the value/volume triangle, which emphasizes a holistic approach to sustainable solutions for producing multiple products by cascading and up-cycling bioresources (Hagman et al., 2018). An illustration of the triangle is provided in Figure 4.1. Green biorefineries are typically cascading the bioresources through extraction and separation methods whereas biorefinery technologies like anaerobic digestion (biogas production), and thermal conversion followed by chemical catalysis or biological conversion can function as upcycling methods of otherwise difficult or low value bioresources.

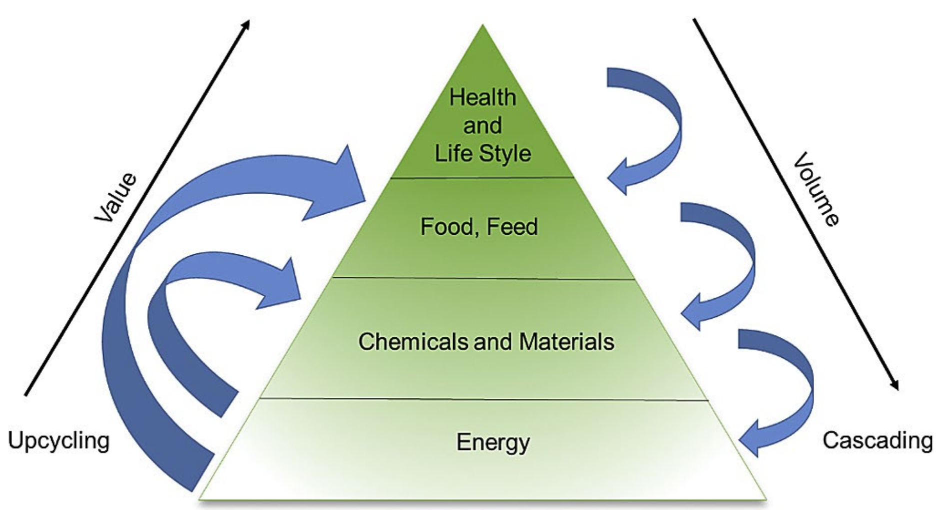

Figure 4.4. Value/volume triangle (pyramid) in biorefineries. Source: (Hagman et al., 2018)

As outlined in the value/volume triangle (Hagman et al., 2018), a combination of e.g. sustainable large-scale energy production typically has to be accompanied by a lower quantity production of a higher valued product. Thus, green energy production often has to be integrated with higher-value side products to provide the same economic stability as seen in conventional oil- and coal-based refineries.

Green biorefineries convert wet green biomass, thus a main process that characterize GB´s is a wet fractionation. Here the wet biomass gets squeezed or pressed and separated into a liquid process stream with soluble plant components and a solid process stream high in fibre content. The GB has an inherent focus on products containing proteins or amino acids, which is due to the high protein productivity of green plants. GB´s can be divided into two types; (i) GB´s processing freshly harvested green biomass and; (ii) GB´s processing storage-stable silage made from green biomass. Silage treatment is a chemical or biological acidification of fresh biomass with the purpose of limiting microbial decomposition and maintaining the nutritional value for ruminants. During the silage treatment and subsequent storage period, large parts of the protein degrades into amino acids, due to enzymatic protease activity and the chemical pH drop.

If the silage treatment is a microbial acidification through lactic acid fermentation, then also the soluble carbohydrate content will diminish, as it is converted to organic acids. The significant difference between the two GB approaches lies therefore mainly in the product possibilities concerning protein/amino acids and soluble carbohydrates/organic acids. GB´s processing fresh biomass will often have a focus on keeping the quality of the plant components as good as possible so this quality can end up in the products. Whereas GB´s processing silage will have a product focus on compounds that are unaffected by the silage treatment (e.g. the lignocellulose fibre) or the amino acids and organic acids themselves. Figure 4.2 shows a schematic overview of a green biorefinery separating wet green biomass into a liquid press-juice and a solid press-cake and outlines different possibilities and examples of product categories from the different process streams. Combining figure 4.1 and 4.2 it is clear that green biorefineries have the potential to produce a diverse set of products with very different applications, economic value, and market volume.

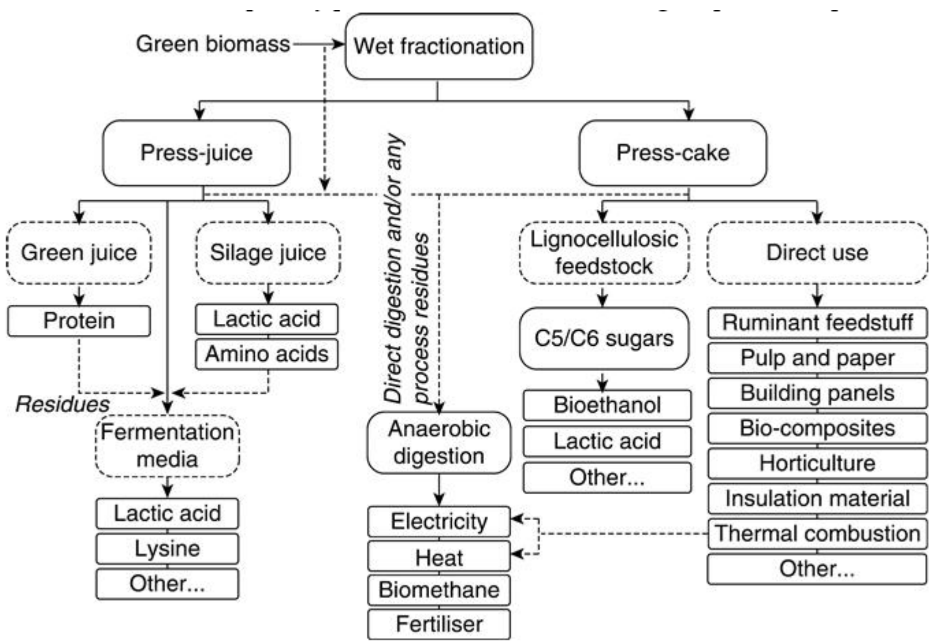

Figure 4.2. Schematic overview of a green biorefinery separating green biomass into a press juice and a press cake, and the product possibilities from the different process streams. (Source: McEniry and O'Kiely, 2014.)

The general GB development in Denmark has focused around the processing of fresh green biomass, with the main product being a protein-rich concentrate that can substitute soy meal in feed mixtures for monogastric animals. This approach will be elaborated in the following chapter. Examples of green biorefineries that process silage instead of fresh biomass include Biowert in Brensbach, Germany (www.biowert.de; IEA Task 37, 2020) BioFabrik in Dresden, Germany (www.biofabrik.com), Newfoss in Uden in Netherlands (www.newfoss.com) and pilot facilities in Utzenaich, Austria (Schaffenberger et al., 2012).

4.2 Green Biorefining protein separation platform

In order to utilize the high protein content of green biomasses for monogastric animal feed, an efficient separation process platform is needed. Several unit operations and steps are involved in the processing of fresh green biomass, before the desired protein concentrate can be separated. The major steps involved are shredding/maceration, fractionation, precipitation and separation. An overview of these process steps and the protein separation platform is presented in Figure 4.3.

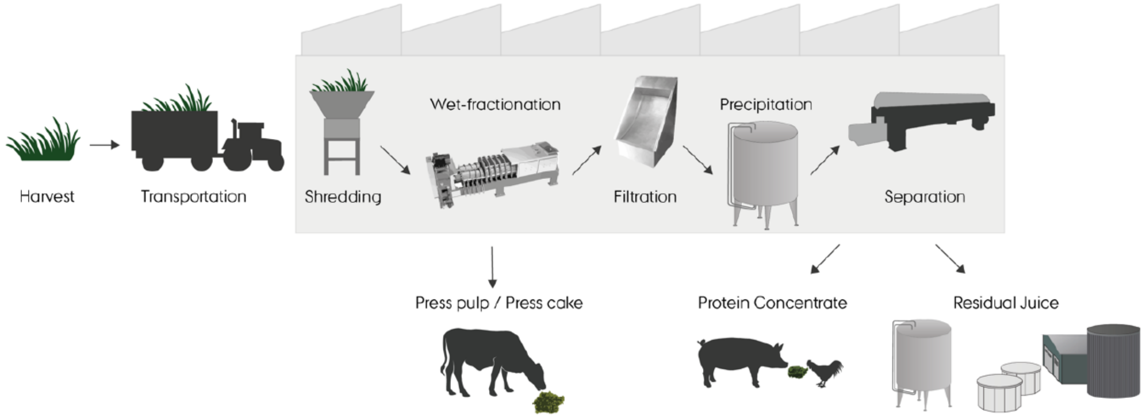

Figure 4.3. The green biorefinery protein separation platform. Unit operations and process steps involved in separation of protein from green biomass in the green Biorefining platform. (Source: Jacobsen, 2020).

The yields and mass distribution between the different processing streams depends on a long list of parameters and can vary to a large extent. Figure 4.4 shows an example of the typical ranges of DM and protein yields following a GB separation process like the one in figure 4.3. Depending on the content and extractability of the protein in the green biomass and the technology and efficiency at the biorefinery, between 50-70% of DM and 40-60% of protein will be retained in the fibre fraction, while the rest is pressed out in the liquid fraction. Following precipitation, 10-20% of the original DM and 20-60% of the original protein can be found in the precipitated protein rich fraction, while the rest will be present in a residual juice (Damborg et al., 2020; Kamm et al., 2010; Ostrowski-Meissner, 1981; Pirie, 1987; and un-published results from L. Stødkilde). These ranges of mass and protein distribution are not ultimate but illustrates the possibilities for optimization of the process according to what the desired outcome is. E.g. if the goal is to have maximum protein yield in the protein concentrate, one has to optimize the fractionation and extract more protein out of the biomass, but also optimize the precipitation and separation to reduce loss of proteins to the residual juice.

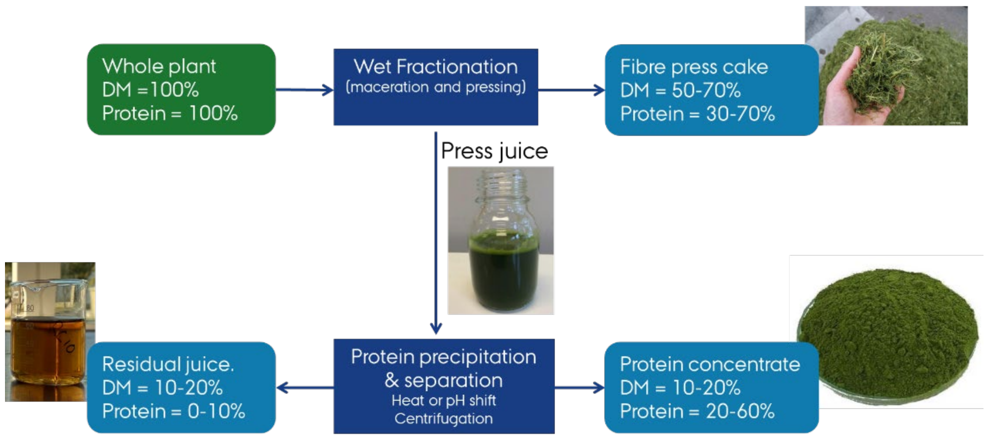

Figure 4.4. Schematic overview and typical DM and protein yields in the fractionation of green biomass into the three process streams of fibre press cake, residual juice and protein concentrate. The numbers are mass balance % (weight per weight in input material) (Source: M. Ambye-Jensen and estimates from Damborg et al., 2020; Kamm et al., 2010; Ostrowski-Meissner, 1981; Pirie, 1987).

The initial step of the processing happens in the field, where the green biomass is harvested. As the entire platform relies on fresh biomass, the harvested biomass is processed immediately, in order to reduce the risk of macronutrient degradation of the desired products (i.e. protein and simple carbohydrates). Immediate processing also reduces the risk of cross-linking between protein and phenolic compounds, which is related to a decrease in protein digestibility (Lærke et al., 2019). This will be further discussed in chapter 5. Furthermore, the harvesting should also aim to avoid sand and soil particles as much as possible, as this will increase the mechanical wear of the process equipment and the soil particles risk to end up in the product streams. Once harvested, the green biomass is transported from the field to the GB. Here, the green biomass is macerated in order to increase the surface area and disrupt the plant cells so the cell content can be pressed out of the biomass more efficiently. This can be done by a number of different machinery types, and include both cutting, shredding and pulping of the biomass. The mechanical pressing of leafy green plants has been studied with varying interest through the 20th century, with some of the biggest efforts being applied during the 1940s and again in the 1970s and 1980s. However, the main technology used for pressing today is screw pressing, which is used in more recent biorefinery pilot and demo plants in Germany (Kamm, et al. 2010), Austria (Kromus et al., 2004; Steinmüller, 2012) and Denmark (Corona et al., 2018, Ausumgaard). The screw press separate the process stream into a liquid and a solid fraction. The liquid fraction is often termed “green juice” for its deep green coloured appearance. This fraction includes the desired soluble proteins and carbohydrates along with free amino acids, enzymes, lipids, inorganic nutrients, and various soluble biomolecules such as tannins and carotenoids etc. The solid fibre fraction, often termed “press-pulp” or “press cake”, is rich in lignocellulose (cellulose, hemicellulose and lignin) as well as the non-separated soluble compounds that is present in the moisture that is left in the press cake. The press pulp normally has a DM content of 30-40% and can efficiently be ensiled directly after the screw press and utilized for ruminant feed, or further biorefined into biomaterials, biofuels and bioenergy.

Following the wet-fraction step, the liquid stream is filtrated, which ensures that the green juice is free from particulates and fibres. The filtrated fibres can simply be recirculated into the screw press and separated once again in the wet fractionation. The next step is the separation of protein from the green juice. Precipitation of the proteins makes way for an efficient subsequent protein separation through centrifugation and has therefore been the main processing route, however proteins can also be separated through membrane filtration (will be discussed in chapter 6). The most commonly used method to precipitate the protein has been by heating the juice to 80-90 °C, which will cause denaturation and coagulation of the proteins. The heating of the juice is often achieved using heat exchangers (Corona et al., 2018; Kamm, et al., 2010) but direct steam injection is also a possibility (Pirie, 1990). The heat denatured and coagulated proteins may then be separated from the juice by centrifugation or decantation. Heat denaturation results in an efficient separation as the denatured protein separates easily out of the solution and results in high protein yields due to fast and efficient processing in heat exchangers. The protein produced by heat treatment will have very low solubility in water, which can be a problem in many food applications, but with regards to animal feed quality heat denaturation has proven successful (Stødkilde-Jørgensen et al., 2021). An alternative to heat denaturation is acid precipitation. In this method, the juice is acidified to reach pH 3.5-4.5, which is close to the isoelectric point of the main protein in leaves, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). At the isoelectric point of the protein, the protein has an overall neutral surface charge and the electrostatic repulsion is low causing the proteins to associate and precipitate. The effect of pH level (pH 3.0 to pH 5.0) on protein precipitation yields from white clover, alfalfa, perennial ryegrass and red clover has been investigated by Damborg et al. (2020), and it was found that the pH level only had a slight effect on precipitation yields in red clover. The acidification may be achieved by adding both inorganic- and organic acids. However, it is also possible to use bacterial fermentation to reduce the pH. The fermentation often uses lactic acid producing bacteria (Ajibola, 1984; Santamaría-Fernández et al., 2017; Santamaria-Fernandez et al., 2019) and the use of lactic acid bacteria have been reviewed by Lübeck and Lübeck (2019). The low pH precipitated protein curd does not sediment as well as the heat denatured due to its soft and hydrophilic properties (Pirie, 1990) and are therefore more difficult to separate out of the press juice resulting in lower yields of protein concentrate. An advantage of using low pH precipitation is a potentially lower energy consumption due to avoidance of heating to 80-90C, however it is important to have an alternative strategy to deal with pathogenic microorganisms from potential soil contamination if the protein product is not pasteurized by heat treatment during processing. Fermentation with lactic acid bacteria could in addition add healthy probiotic effects of a following feed product (Lübeck and Lübeck, 2019). The fermentation temperature (37 °C) and fermentation time (app. 6-8h) will result in some degradation of the proteins and there will be no native RuBisCO left after fermentation (Ameenuddin, 1983; Nissen et al., 2021.).

Instead of concentrating the protein by either heat or acid precipitation, it is also possible to concentrate protein from the juice by membrane filtration. This method generally uses membranes with pore sizes that allows permeation of water and smaller compounds while retaining proteins. Ultrafiltration (membrane pore size lower than 0.1 μm) of protein from leaf juice has been shown to produce similar yields as heat denaturation (Castellanos et al., 1994; Koschuh et al., 2005; Ostrowski-Meissner, 1980), however similar results have been difficult to reproduce in the lab at AU Biological and Chemical Engineering (BCE) in Foulum (N. Hachow Motta dos Passos et al., Manuscript in prep.). Zhang et al. (2015) tested different microfiltration (pore size larger than 0.1 μm) and ultrafiltration systems for concentration of leaf protein and found that microfiltration was the most efficient method. This was unexpected, since the microfiltration should allow most proteins to pass through the membrane, but similar to results at AU BCE in Foulum (N. Hachow Motta dos Passos et al., Manuscript in prep.), and it shows that proteins may easily be retained due to membrane fouling. When performing membrane filtration, the filtration time and temperature may affect the amount of native protein and significant degradation of RuBisCO has been observed when performing the filtration at room temperature (Koschuh, et al., 2005).

Upon precipitation, the final liquid/solid separation is applied, typically using a decanter centrifuge. The centrifugation produces a moist solid fraction of about 40-50% DM of the protein concentrate, which contains the precipitated proteins together with other plant constituents such as lipids and carbohydrates that have precipitated out together with the proteins. But also soluble nutrients and biomolecules present in the moisture content of the moist solid fraction. The liquid fraction is a residual juice often termed “brown juice” and contains the remaining soluble compounds, such as oligo- and mono-carbohydrates or organic acids (in case of fermentation), free amino acids, inorganic nutrients etc. Since this fraction contains compounds easily converted by microbial digestion, it is often used for input in anaerobic digesters for biogas production (Feng et al., 2021; Santamaria-Fernandez et al., 2018), but may also be the input of membrane filtration systems and more refined separation of specific compounds.

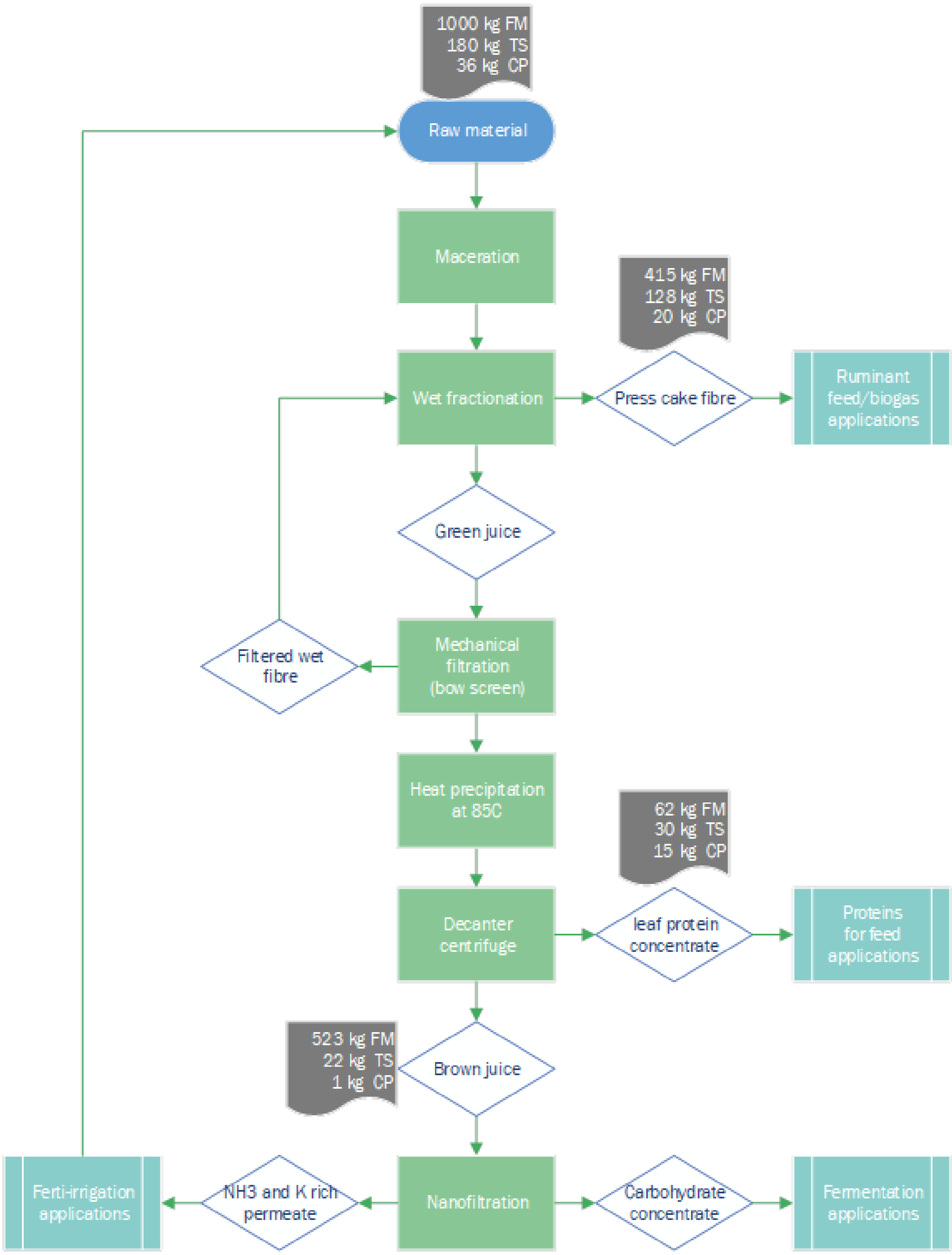

Figure 4.5. Process flow diagram of a green biorefinery with focus on producing a press cake for ruminant feed or biogas production, a leaf protein concentrate for monogastric animal feed and a residual brown juice for biogas production and nutrient recirculation. Green boxes are unit operation processes. Diamond squares are process streams. Turquoise boxes are product applications. Grey labels include an estimated mass balance with amounts of FM:fresh matter, TS:total solids, CP:crude protein in input and output streams

As previously stated, many products can be derived from this GB protein separation platform. Thus, additional downstream processing can be performed in order to differentiate the desired end-products, depending on the targeted market (e.g. feed, food, biomaterials, biofuels, bioenergy etc.). However, the major steps involved in a base case protein separation green biorefinery have been elucidated above. Figure 4.5 shows a process flow diagram over the described process and includes a theoretical estimate of DM and protein distribution between the three process streams. The estimate is based on lab scale experiments and pilot- and demo-scale experiences at the AU Foulum platform for GB and estimated to be realistic in an optimized and continuous production. Here 16% of the DM and 42% of the protein ends up in the leaf protein concentrate while 71% of the DM and 56% of the protein ends up in the press cake fibre, the rest of both DM and protein ends up in the residual juice. The mass balance is a constant subject of optimization and some of the specific developments to optimize the protein yield into the protein concentrate is described below.

4.3 Status of the Danish base case with focus on feed and biogas

The Danish base case green biorefinery is the term, here used for the general GB focused on the processing of fresh green biomass into a protein-rich concentrate that can substitute soy meal in feed mixtures for monogastric animals, a press cake fibre for ruminant feed and/or biogas production and a residual juice for biogas and nutrient recycling. There has been a substantial research and development effort over the past 8 years in Denmark on this case, starting from the pioneering projects BioValue SPIR (Innovationsfonden, 2013-2018), Biobase (AU & DCA, 2014-2017) Organofinery (Organic RDD, 2014-2017), and Multiplant (Organic RDD, 2014-2017). At present more than 25 R&D projects related to the green biorefinery have been funded (see chapter 10). All Danish universities are involved in different aspects of the research and development together with the large knowledge institutions and a long list of active industrial partners. Two R&D platforms for upscaling, tests and process developments in pilot- and demoscale have been established at Teknologisk Institut in Tåstrup and at Aarhus University in Foulum, respectively. Industrial initiatives have at present resulted in two new commercial demonstration plants in operation spring 2021, and more are in the planning phase.

The development is going fast, but there is still many issues to be solved and to be further investigated before a novel green biorefinery industry can thrive in Denmark, as well as beyond the borders in EU and all other places with suitable climate for good availability of green biomass. Such issues are included in the list below:

- Plant breeding for optimal protein extractability and quality
- Development of efficient management for harvest and logistics to deliver good quality green biomass to the biorefineries
- Process development and optimization at the biorefineries to get constant high yields of protein concentrates with constant high digestibility and nutritional value

- Value creation of the press cake fibre and the residual juice to achieve a better overall business case for the green biorefinery
- Technology integration for cascade utilization of side streams and residues
- Development of flexible biorefinery solutions, where input biomass and output products can change according to seasonal variation, in order to achieve efficient use of production facilities all year round.

The following chapters give examples of R&D activities and the associated projects, which try to solve and investigate the issues listed above.

4.4 Examples of optimization along the green biorefinery value chain

Screening and selection of plant varieties that yields highest amounts of extractable protein is a focus area in both the GUDP project AlfaMaxBioraf (2019 -2022), where screening and selection is carried out on alfalfa species, and the GUDP project Græs-prof, where it is carried out on more than 300 different grass and clover species. In both projects the screening activity is led by the Danish seed company DLF.

Tests of harvest methods and logistics for better understanding of the consequences on processing yields and product quality, as well as economical optimisation of the supply chain is a key focus area in the development. For the Danish base case scenario that utilize freshly harvested green biomass in order to produce a protein concentrate suitable for monogastric animal feed, it is very important to secure a fresh quality of the input biomass at the biorefinery. Biological deterioration, degradation and cross binding of the protein and other reactive plant components kick in at the moment the biomass is harvested. This degradation and cross binding put restraints on how much time, from harvest to processing, that can be accepted in order to reach a certain yield and quality of the protein concentrate. As these biological processes are affected by several parameters including biomass type and maturity, temperature, amount of cutting/maceration in the field, it is an important point that the accepted time between harvest and processing will change over the season and depend on the management operations in the field. There is very little research on the issue of time between harvest and processing, but early studies showed yield reductions of up to 50% after 9 hours at 25 °C and 35 °C (Bart et al., 1976; Pirie, 1987). New investigations into this are being carried out in several of the ongoing projects with Danish participants. At the Demoplatform for GB at AU Foulum, it has been the observation, over the course of pilot and demo scale development, that yield and quality increases by fast processing, but exactly what fast means is now being tested in large demo scale experiments in the projects Green Valleys (Interreg, 2018-2021) and GO-GRASS (H2020, 2019-2023). The first demo scale harvest/time experiment was conducted September 2020 and showed no or only little yield reductions after processing between 1 hr to 8 hr after harvest, and the protein concentrate had the same content of crude protein of around 50% of DM (no other quality parameters were measured). However, 24 hr after harvest the protein yields reduced to 1/3rd of the yield at 1 and 8 hr after, and the protein content dropped to 35% of the DM (preliminary data from the AU Demoplatform, M. Amby Jensen 2021) This study was carried out at one set of conditions (15 °C, grass clover mixture no. 42, and relatively early cut - only 10-15 cm tall), it is thus only a snapshot of the conditions that would represent a whole seasonal operation in DK. New experiments are planned in 2021 in the same projects as well as within the GUDP project Græs-prof where partners from the first commercial farm-scale green biorefinery in DK, Ausumgaard, are participating. The results from this development will be integrated into more specific planning and organizing of the total harvest and logistics planning and techno-economic assessments of those. Two examples of studies looking at the techno economics of the supply chain green biorefineries are Hoeltinger et al. (2012) looking into an Austrian case of GB and O´Keeffe et al., (2012) looking into an Irish case of GB. However both with main focus on processing silage grass and therefore less constrained on the fresh quality.

The yields of different process streams and specific products are always an important part of process optimization. As previously discussed and highlighted in figure 4.4 the yields can vary to a large degree and depends on many variables. While the yields presented in figure 4.5 are fairly easy to achieve in controlled lab-scale experiments, it has proven much more challenging in large pilot and demo scale. The experience from the Demoplatform at AU Foulum shows that while it is sometimes possible to reach yields of protein concentrates above 15% of total solids input and 35% of total protein input, it is not the normal case until now. Much of the yield losses can be assigned practical losses of biomass from weighing-in input material to the product output. The optimisation here is primarily about handling and operational optimisation. After the first full seasonal operation in 2020 at the AU Demoplatform yields have already been improved and operation is much more consistent. However, this kind of operational optimisation is much more applicable in continuous production facilities than in an experimental test and development platform like the AU Demoplatform. It is therefore expected that commercial facilities will be able to optimise this much further. However, it has still become clear that yields of protein concentrate and especially protein yields in this product chain needs to be given attention in order to reach the assumed mass balance. The before mentioned harvest and logistic optimisations add to this, but also better processing can increase the yields. Test and optimisation of maceration and pressing for higher juice yields is carried in both the GUDP Græs-prof project, and in the Green Valleys Interreg project. The results point towards a need for efficient maceration before the pressing in order to achieve steady yields of around the ones estimated in figure 4.5. In Nov 2020 a more severe maceration was tested and compared to the shredding and cutting that has been tested so far. The results are preliminary and only repeated a few times, but gave protein concentrate yields of 17% of the total solids input and 40% of the total crude protein input (preliminary data from the AU Demoplatform, M. Ambye Jensen, 2021). Further testing of more severe maceration including disrupter type maceration known from the biogas industry will be carried from 2021.

4.5 Examples of products from fibre pulp

The press cake or fibre pulp coming out of a green biorefinery constitute the major part, around 70%, of the total solids output. It is therefore highly important that this huge bioresource has a relevant and valuable application. In the Danish base case it is tested as either ruminant feed or as solid substrate for anaerobic digestion and biogas production. Both applications are very relevant in an integrated agricultural system where the green biomass often comes from dairy farmers that need feed for their livestock and for the biorefinery that need renewable energy in order to have a sustainable production (Djomo et al., 2020).

However, the fibre pulp has numerous other applications and the possibilities for adding further biorefining technologies are many.

In the GUDP project Grass Biochar (2020-2023) it is investigated how GB can be integrated with pyrolysis of the fibre pulp. The pyrolysis will produce renewable energy to supply the heat for protein coagulation and drying of the protein concentrate, as well as a high quality biochar or activated bio carbon. The bio carbon is an extra product with applications as feed ingredient where biochar is used to increase animal health and digestion. The project has until now screened different types of fibre pulp for its qualities in the pyrolysis process. Next step is to integrate the company Aquagreen´s demo scale pyrolysis test rig to the Demoplatform green biorefinery at AU Foulum. Large scale production of biochar from the grass fibre will open up significant potentials for creating Bioenergy with Carbon Capture and Storage (BECCS) solutions in combination with green biorefineries.

Using the fibre pulp for fibre based biomaterials is another valuable application. This approach is in fact the main aim for all of the existing GB´s that process silage instead of fresh green biomasses. Biowert in Brensbach, Germany (www.biowert.de) produces grass based insulation material and grass fibre enforced bioplastic, a biocomposite material suitable for injection moulding or extrusion applications. Newfoss in Uden in Netherlands (www.newfoss.com), produces insulation materials and fibres for paper and packaging. The GUDP project SinProPack (2020-2023) in DK has recently started the investigations and development of producing biobased packaging for the takeaway market from the press cake fibre pulp from green biorefineries. The GUDP project Høsttek (2021-2024) has likewise just started developing sustainable fibreboards of the press cake fibre.

Similar to the packaging application where it is the content of cellulose that is in focus, the cellulose from the fibre pulp can also be a source of cellulose for biotextile manufacture. In on-going work at AU BCE the press cake fibre is pulped, solubilised and regenerated into cellulose based biotextiles. This work is so far still preliminary and unpublished, but it is described in a popular science article at Videnskab.dk (https://videnskab.dk/teknologi-innovation/dansk-forsker-laver-tekstil-af-graes-er-det-fremtiden).

An alternative use of the fibre pulp can also be found in direction of the lignocelluloses sugar platform biorefinery. Developed for primarily wood and agricultural residues and for the production of primarily bio-ethanol, lignocelluloses biorefineries consists of pre-treatment, enzymatic hydrolysis and fermentation for biobased chemicals or biofuels (de Jong, 2009).

Common to both fibre pulp utilisation for biomaterials and for lignocelluloses sugar platform biorefining is that it is an advantage if the fibre is depleted of its protein content. Thus efficient extraction of protein at the green biorefinery pose no negative impact on these applications. However, for the application where the fibre pulp is utilized for ruminant animal feed, there is a lower limit of how little protein should be left in the pulp.

4.6 Examples of products from residual juice

The residual juice after protein precipitation and separation constitutes around 10-20% of the DM input, but typically over half of the fresh weight input. The process stream is therefore a significant part of the green biorefinery outputs but is mostly water with a low concentration of solids. The specific composition of different residual juices are dependent on a number of factors including both the processing steps involved in the GB separation platform, especially the precipitation method, as well as type-, maturity- and growth conditions of the green biomass input.

The application for anaerobic digestion (AD) of the residual juice is a straightforward opportunity, especially in DK, which has a significant biogas industry. Many of the biogas plants in DK could benefit from an extra substrate with low, but easily digested, solids concentration in order to co-digest more fibrous agricultural residues such as deep litter, cow manure and straw from cereal grain and grass seed production. This is for example the case at Ausumgaard, the first commercial green biorefinery in DK (https://ausumgaard.dk/baeredygtig-energi/graesprotein/), which have a large biogas facility where both the residual juice and the fibrous pulp coming out of the biorefinery can be digested. The use of residual juice for AD have been evaluated both in terms technical, economic and environmental sustainability (Corona et al., 2018; Djomo et al., 2020; Feng et al., 2021; Jensen and Gylling, 2018; Santamaria-Fernandez et al., 2018). In an evaluation report, Jensen and Gylling (2018) discuss the economic perspectives of value chains in GB. Here, it was concluded that the use of residual juice as a substrate for biogas production yields an overall negative revenue. The main reason for the negative revenue is the expected costs for handling and transportation of the substrate. An obvious suggestion to reduce the transportation costs would be to implement a GB protein separation platform near an already existing biogas facility or include the construction of a new biogas facility in immediate vicinity. If the residual juice cannot be co-digested in an existing AD plant, it is a much cheaper and efficient solution to install a packed bed reactor, as shown by Feng et al. (2021). Here residual juice was efficiently digested as a sole substrate at low retention time (5.5 days) and therefore a much smaller reactor size and capital investment is needed.

An obvious advantage for AD of the residual juice from green biorefineries is that the inorganic nutrients will be led directly into an existing recirculation of nutrients as the digestate from AD is spread back on agricultural land as fertilizer, already in the current system.

However, the residual juice could potentially be used for much more than bioenergy, before nutrients is recirculated back to the agricultural production. Historically, the focus of valuable products from residual juice/brown juice from green biomass processing, has been around amino acids and lactic acid. Several studies and commercial activities have looked into the production of amino acid concentrates (Ecker et al., 2012) or specific amino acids such as L-Lysine (Andersen and Kiel, 2000; Thomsen et al., 2015). L-Lactic acid is a low molecular weight commodity biochemical with primary application in biobased PLA (polylactic acid) plastic materials. It is produced in bulk quantities with an estimated 1m tonne production in 2020 (Nova Institute). L-lactic acid has been the primary target for value added products from brown juice both from green pellet drying industry (Andersen and Kiel, 2000) and from GB setups where lactic acid fermentation is a mean for protein precipitation (Lübeck and Lübeck, 2019; Santamaria-Fernandez et al., 2020). Both amino acids and L-lactic acid needs to be separated by membrane filtration and delicate purification methods including ion exchange (Ecker et al., 2012).

In the few existing green biorefineries which are processing silage grass, the juice is used for bioenergy through biogas production (Biowert) or its amino acid, organic acids and inorganic nutrient content is used as primarily fertilizer products, which is concentrated through membrane filtration technology ().

When processed in the Danish base case setup, shown on figure 4.5, the residual juice will be high in carbohydrates and inorganic nutrients. This combination has high potential for making a good substrate for fermentation applications in the biotech industry. However, the shift in process design of the GB platform also comes with a need for development of novel process design implementations in order to obtain a residual juice rich in carbohydrates suitable for fermentation. Moreover, different residual juice compositions might require different microorganisms for the best utilization of available nutrients. However, the present (i.e. the last 20 years) literature on fermentation research of residual juice have all included lactic acid bacteria as a facilitator for either preparation of residual juice to a subsequent lactate consuming fermentation, or as a producer of lactic acid as the end product (Andersen and Kiel, 2000; Bákonyi et al., 2020; Lübeck and Lübeck, 2019; Thomsen and Kiel, 2008; Weimer and Digman, 2013).

Figure 4.6 shows examples of di-and monosaccharide composition and total concentration in residual juice from different biomasses processed at the Demoplatform in AU Foulum. The total di- and monosaccharide concentration varies from 7-22 g/L in the juices and it can be seen that while the juices from grasses contain primarily fructose and glucose, the legume juices also contain significant amounts of sucrose and xylose.

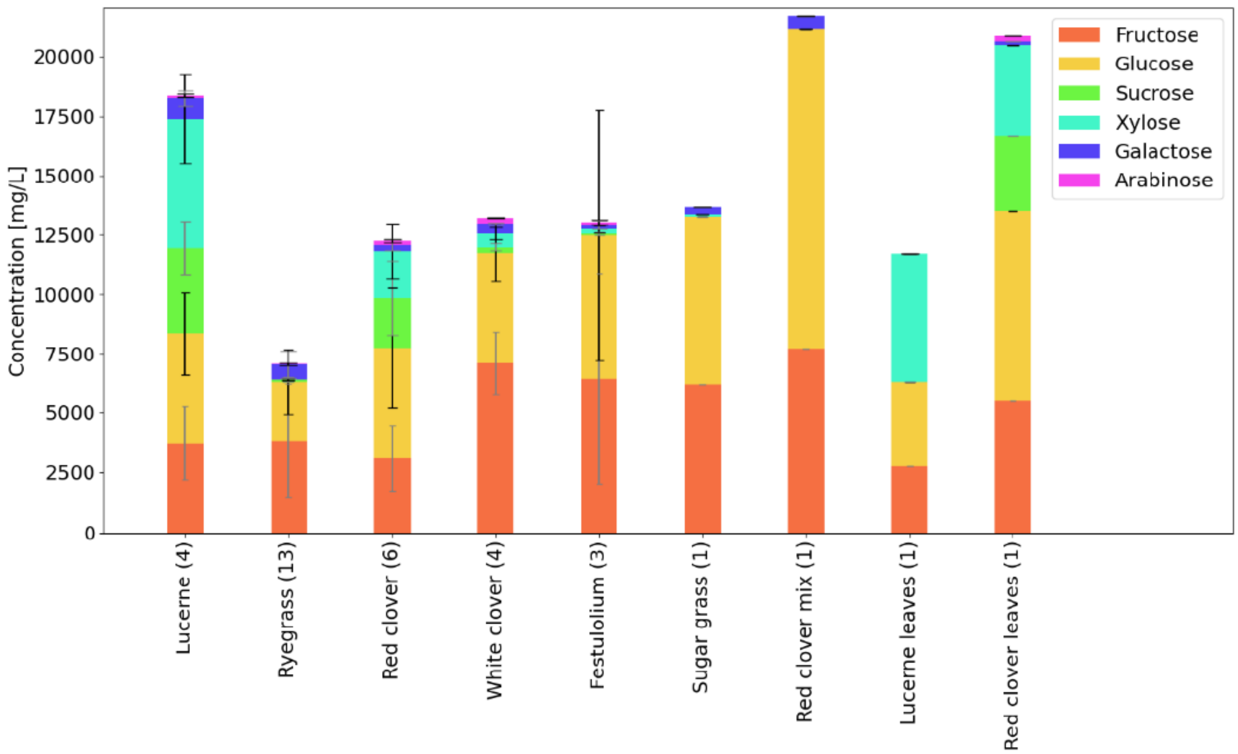

Figure 4.6. Di-and monosaccharide content and distribution in residual juice from the Demoplatform at AU Foulum (compiled process data from operation and tests during 2019 and 2020). For each different biomass the data is compiled, and the figure shows the average of a varying number of tests for each biomass. The number of tests that is shown in brackets. (Source: M. Ambye-Jensen, manuscript in prep.)

In order to achieve a good fermentation substrate, it is an advantage to reduce the volume and increase the concentration of the carbohydrates as well as other macronutrients present in the residual juice. This is carried out by membrane filtration.

In an ongoing project funded by the Promilleafgiftsfond (Opskalering og validering af processer for separering af restsaft fra produktion af græsprotein), AU BCE is developing demoscale nanofiltration methods to upconcentrate the residual juice and produce a concentrate and a permeate from where an example is shown on the picture in figure 4.7. It is in general feasible to reach a volume reduction factor of 15 and a total di-and monosaccharide content of 30-60 g/L (G. Tirunehe, manuscript in prep). Examples of biobased chemicals/products that have been produced from this concentrated residual juice include ethanol, astaxanthin and single cell protein (MSc Thesis Bodil Hinge Jepsen, 2021, MSc Thesis Emil Jacobsen, 2020, MSc Thesis Peter Schultz, 2021). All examples have only been studied in student thesis´ and more work is needed to take the development further.

The permeate is on the other hand a clear liquid containing only elevated concentrations of potassium- and nitrate salts. The project investigates the qualities of this permeate for ferti-irrigation applications.

Figure 4.7. Picture of the two outputs from nanofiltration of residual juice. Left is the permeate going through the membrane. Right is the retentate, which is retained by the membrane. (Source G. Tirunehe, manuscript in prep)

Table 4.1. Composition example of heat precipitated brown juice from grass as input biomass, before and after membrane separation. ND = Not Detected, NM = Not Measured. (Source G. Tirunehe, manuscript in prep)

Brown juice composition prior to / after membrane filtration								
Process Stream	Volume (m^3)	Brix (°Bx)	Glucose (mg/l)	Fructose (mg/l)	Galactose (mg/l)	Sucrose (mg/l)	Citric acid (mg/l)	Succinic acid (mg/l)
Feed Brown Juice	2.03	2.66	2,469	2,452	128	128	NM	NM
Concentrated Brown Juice	0.048	19.2	8,872	12,758	776	527	NM	NM
Feed Brown Juice	2.55	3.2	3,099	2,879	154	836	NM	NM
Concentrated Brown Juice	0.22	16.7	9,667	15,601	634	1,270	NM	NM
Feed Brown Juice	1.91	3.65	6,156	2,695	ND	536	NM	NM
Concentrated Brown Juice	0.18	18.5	19,925	8,535	ND	1,810	NM	NM
Feed Brown Juice	2.03	4.0	6,938	4,770	226	1,871	1,103	278
Concentrated Brown Juice	0.12	15.7	22,371	13,115	272	16,386	3,467	546

Additional work in membrane filtration within the green biorefinery setup, is the possibility of separating soluble protein by ultrafiltration instead of separating by precipitation and centrifugation separation, or by precipitating at lower degrees (50-60 °C), centrifuge the precipitate, and membrane filtrate out the rest of the soluble proteins (primarily RuBisCO). This will be further discussed in chapter 6 on proteins for food. Adding these different options for processing at the green biorefineries, the process scheme could instead look like on figure 4.8. In this way the value creation can be optimised significantly through more additional separations and sophisticated separation technology.

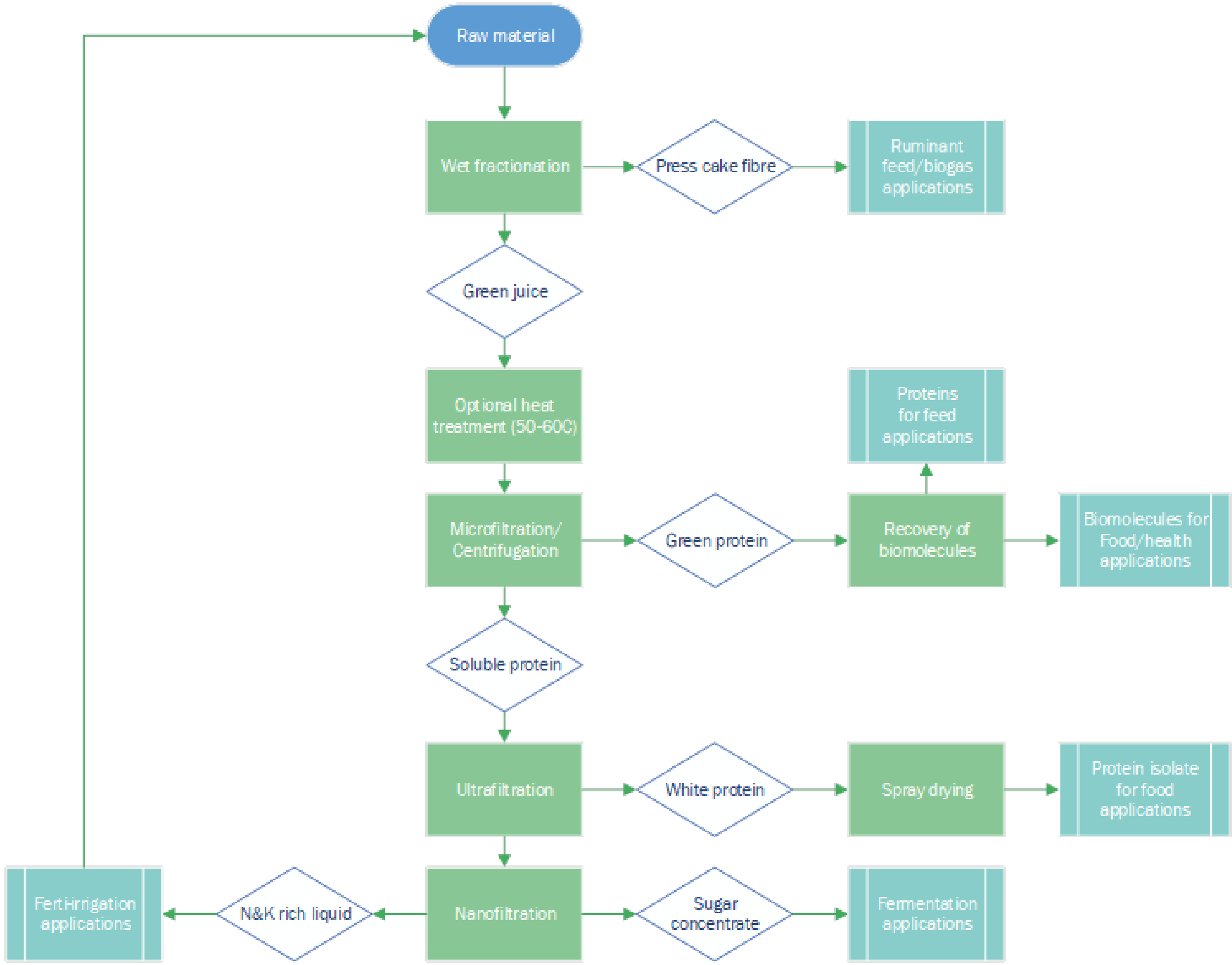

Figure 4.8. Process flow diagram of a green biorefinery with possibilities for creating higher value products by separating the protein in two fractions (green and white protein), separating colorants or other biomolecules for ingredients and up-concentrating sugars for fermentation applications. Green boxes are unit operation processes. Diamond squares are process streams. Turquoise boxes are examples of product applications.

5 Feeding value

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5.1 Proteins for monogastrics

5.1.1 Chemical composition

The nutritional quality of plant juice and protein-rich precipitate has been investigated in several studies with monogastric animals. Most studies were performed before 1990 with alfalfa as input biomass and digestibility and growth performance were main parameters evaluated. The outcome of these experiments was inconclusive (Houseman & Jones, 1978; van der Heide et al., 2021), mainly due to variations in the methods used and to improper processing (Chiesa & Gnansounou, 2011; Houseman & Jones, 1978). However, improvements in methods for green plant processing and protein extraction methods combined with the increasing demand for more sustainable livestock production fuel new initiatives to produce high quality protein alternatives to soy protein for animal feed. In the more temperate climates in Northern Europe, perennial crops such as clover and grasses have gained increasing interest as input biomass for biorefining. The crude protein (CP) content of the extracted protein is highly dependent on process parameters (Chiesa & Gnansounou, 2011; Davys & Pirie, 1965) and varies between studies (Table 5.1). Despite the variations in CP content, there is limited variation in amino acid composition between species and studies. The conserved enzyme RuBisCO constitutes the majority of protein in plant leaves and therefore also in the extracted protein (Pirie, 1978) resulting in a relatively stable amino acid composition. The amino acid profile of proteins extracted from green biomass is generally very similar to soybean meal and the content of several of the essential amino acids is also higher than corresponding values in soybean meal. A particular benefit is the higher proportion of the essential amino acid methionine (relative to lysine) in the green products compared to soya bean meal, which particularly in organic poultry production makes it easier to fulfil the nutritional requirement. Moreover, protein extracted from legumes, grass and the grass-clover mixtures has a high content of threonine (relative to lysine) and can supply this amino acid to a diet with limited content such as maize-based diets (Russell et al., 1987).

During the biorefining process, plant lipids are concentrated together with the extracted protein (Pirie, 1978) resulting in a crude fat content of up to 14% (Stødkilde et al., 2020). The majority of the fat are unsaturated fatty acids and dominated by the omega-3 fatty acid alpha-linolenic acid. Unsaturated fatty acids of the omega-3 and -6 family are essential fatty acids and thus of nutritional importance. On the other side, unsaturated fatty acids are susceptible for oxidation and precautions are necessary to avoid rancidity (Stødkilde et al., 2020). Data on the composition of mineral in the extracted protein is limited. However, parameters during processing are suggested to influence the mineral distribution between fractions and hence also the mineral content in the extracted protein (Baraniak, 1990). Extracted protein from alfalfa is characterized by a high content of macro- and microelements, generally exceeding the values in the alfalfa plant. However, precipitating the proteins with acidification led to a low content of calcium and potassium, likely due to the minerals being distributed to the brown juice (Baraniak, 1990).

Table 5.1. Amino acid composition in protein extracted from white clover, red clover, alfalfa, perennial ryegrass and grass-clover under Danish conditions (g/16 g N).

Amino acid	**White clover[1]**	**Red clover[1]**	**Red clover (elongation stage)[2]**	**Red clover (flowering stage)[2]**	**Alfalfa[1]**	**Perennial ryegrass[1]**	**Grass-clover[3]**	**Grass-clover[4]**	**Grass clover[5]**	**Soybean meal[6]**
CP (g/100 g DM)	34.7	34.3	41.5	35.0	38.8	24.0	36.2	45.8	56.2	43.9
Lysine	6.3	6.7	5.7	5.5	6.6	5.6	5.7	5.8	5.7	6.3
Methionine	1.8	1.9	1.7	1.6	1.9	2.1	1.8	2.3	2.0	1.4
Cysteine	0.8	0.8	0.5	0.4	1.1	0.9	0.7	0.5	0.7	1.6
Threonine	5.0	5.0	4.3	4.3	5.0	4.8	4.2	5.0	4.6	4.0
Histidine	2.3	2.4	2.2	2.1	2.5	2.1	2.0	2.1	2.1	2.9
Isoleucine	5.4	5.5	4.8	4.6	5.4	5.1	4.7	4.6	4.5	4.5
Leucine	9.1	9.3	8.2	8.0	9.0	8.9	7.7	8.9	8.6	7.8
Phenylalanine	6.1	6.1	5.6	5.1	6.1	6.0	5.0	6.0	5.6	5.2
Valine	6.6	6.8	5.8	5.7	6.5	6.5	5.9	5.9	5.6	4.4
Arginine	6.0	6.1	5.5	5.2	6.2	5.9	5.1	6.0	5.7	7.2
Serine	4.9	4.9	3.9	4.1	5.0	4.6	3.9	4.4	-	4.9
Proline	4.9	4.9	3.9	4.4	4.7	5.0	3.9	4.9	-	5.5
Alanine	6.4	6.4	5.0	5.2	6.0	7.2	6.6	6.5	6.0	4.4
Glycine	5.6	5.6	4.4	4.6	5.4	5.7	5.0	5.6	-	4.3
Asparagine/Aspartic acid	12.0	11.5	9.7	10.1	12.9	9.2	8.6	9.6	-	11.1
Glutamine/Glutamic acid	11.1	11.3	9.2	9.7	10.9	10.3	9.6	11.0	.-	17.7

[1] Stødkilde et al., (2019). 2 Santamaría-Fernández et al., (2019). 3 Stødkilde et al., (2020). 4 Stødkilde et al., (2021). 5 Vils et al., (2020). 6 Soybean meal, Solvent extracted, CP 43.90% (National Research Council, 2012).

5.1.2 Digestibility

Currently several Danish projects within biorefining of green biomass are ongoing or finalized. Initial digestibility experiments with rats were performed with extracted protein with a relative low protein content (35-40% of DM). They showed digestibility of protein up to 85% and at the same time they revealed a clear positive correlation with the protein content in the protein concentrate (Stødkilde et al., 2018, 2019) emphasising the importance of directing process optimisations towards increasing protein content in the concentrate. This is further highlighted in a recent rat study, where alfalfa protein with up to 72% CP in DM showed a protein digestibility of 91%, which is similar to high quality soy concentrate used for weaning piglets (Stødkilde et al., 2021, in manuscript). Combined, the rat studies also demonstrate that screw-press processing does not induce major quality impairing changes in proteins with respect to digestibility in monogastrics.

The ileal digestibility of protein and amino acids was determined in cannulated pigs using protein extracted from red clover and perennial ryegrass. The protein was produced as a first generation product on a pilot facility under development resulting in a product with a low CP content (30%). This was reflected by a digestibility that for all amino acids was significantly lower than in soybean meal, likely as a result of a high ash and fibre content (Lærke et al., 2019). Similar to the rats, the results highlight the importance of focusing on increasing protein content through optimizations of the process. The ileal digestibility of amino acids in protein extracted from alfalfa, red clover and festulolium through an optimized biorefining process is being evaluated in an ongoing project. The protein has been extracted using different methods and CP contents of >62% have been reached in the most refined products. Results are expected in 2021 and will provide important information for feed formulations.

5.1.3 Effect on monogastric performance

The first Danish production experiment was performed with a relative low protein containing concentrate containing 36% CP. With this protein concentrate it was possible to substitute 8% of the diet, primarily soy press cake, (13% of the CP) for organic broilers with protein concentrate extracted from organic grass.-clover without affecting growth performance (Stødkilde et al., 2020). However, larger inclusions challenged feed intake and growth rate due to the low protein content and the correspondingly high content of indigestible fibres in the protein extract. Protein from the same production was used in a trial with organic layers, where 0, 4, 8, and 12% of the feed was substituted with the grass-clover protein. The study demonstrated good palatability and inclusion of grass-clover had no effect on egg production, egg weight, strength of eggshell, or feed utilization (https://icrofs.dk/aktuelt/nyheder/nyhed/artikel/kloevergraes-som-foderprotein-til-oekologiske-aeglaeggere). The second production experiment was performed with growing-finishing pigs with a protein concentrate containing 46% protein. With this protein content it was possible to substitute at least 15% of the traditional feed (up to 41% of the crude protein) with grass-clover protein and still obtain feed intake, growth and feed utilisation similar to a control group with soy as the dominating protein source (Stødkilde et al., 2021). In the experiment, soy was completely eliminated from the finishing mixture when 15% grass-clover was included. All pigs performed equally, and no adverse health effects or meat quality changes were observed by increasing grass-clover protein levels in the feed.

The third production experiment was performed in collaboration with SEGES Pig Research Centre, Grønhøj in a conventional experimental setup (Vils et al., 2020). In this experiment "green protein" with 56% protein was included in a feed mixture for growing-finishing pigs with local protein sources (faba beans and rape-seed cake) and compared to faba beans and rapeseed cake alone as well as and a traditional protein mixture consisting of soybean meal and sunflower. A slightly higher feed intake but lower feed utilization of the finishing mixture with green protein was observed. The production results (biological response to feed mixtures) were generally high and did not differ between groups. Also here, no negative effects on animal health were observed. The recent results on pig performance confirms early studies (Sugimoto et al., 1986) where soy was substituted 100% by alfalfa protein without affecting the general feed intake, weight gain and feed conversion ratio. Similar positive results were observed by Pietrzak and Grela (2015), where sows in late lactation had a higher weight, when alfalfa protein concentrate (1.5%-3.0%) was included in the diets.

As with the digestibility, process parameters will affect performance exemplified by the higher performance in pigs fed freeze-dried alfalfa green protein than in pigs fed commercial alfalfa protein concentrate (X-Pro) produced by drying at higher temperatures (Cheeke et al., 1977). These challenges are also the focus of the before-mentioned and ongoing rat and pig digestibility trials (Stødkilde et al., manuscript in prep).

In meat products from broilers and pigs fed protein extracted from grass-clover, the high content of unsaturated fatty acids in the extracted protein is reflected in the fatty acid composition of the meat (Stødkilde et al., 2020, 2021). Increasing the dietary content of grass-clover also increases the content of alpha-linolenic acid in the meat resulting in an increased unsaturation of fatty acids. As a result, the shelf life can be affected and this needs to be investigated in future studies.

Feeding broilers with grass-clover protein deposits a clear yellow colorization in both meat and fat as a result of carotenoids in the protein (Stødkilde et al., 2020), similarly a significant effect was seen on the yolk colour of grass-clover protein fed layers https://icrofs.dk/aktuelt/nyheder/nyhed/artikel/kloevergraes-som-foderprotein-til-oekologiske-aeglaeggere/. Including 15% grass-clover protein in the diets for growing-finishing pigs darkens the meat (M. longissimus lumborum, M. biceps femoris) compared to control (Therkildsen et al., 2019). Substituting soy for grass-based protein has no effect on the sensoric profile of the meat (taste, flavour, texture) (Therkildsen et al., 2019; Vils et al., 2020).

5.2 Fibre feed for ruminants

5.2.1 Chemical composition and in vitro digestibility

For ruminants, the main focus is on evaluating the feed value for the fibre-rich pulp fraction originating after screw pressing of the green biomass. Around half of the plant crude protein will distribute to the pulp, and the composition of amino acids in this fraction is similar to the composition in the whole plant (Damborg et al., 2018). As a considerable proportion of the protein retained in the pulp is fibre-associated, the pulp is expected to be suitable for ruminants. Chemical analysis of the pulp revealed a fraction with a higher DM concentration than the plant, similar crude protein concentration and lower crude ash concentration (Table 5.2) (Damborg et al., 2018). The *in vitro* digestibility tended to be lower for the pulp, as expected due to a large proportion of soluble organic matter being removed upon juice extraction. When expressed as digestible organic matter (DOM) as proportion of DM, though, no major difference was observed, because the ash content is also reduced during the extraction step.

Table 5.2. Chemical composition of red clover, perennial ryegrass, alfalfa and white clover plant and pulp.

Plant Species	Fraction	DM (g/kg)	Crude protein (g/kg DM)	Crude ash (g/kg DM)	In vitro OM digestibility (g/kg OM)	DOM (g/kg DM)
Red clover	Plant	166	205	90.6	654	594
	Pulp	435	198	66.3	579	540
Perennial ryegrass	Plant	199	167	86.3	744	679
	Pulp	414	164	51.1	699	663
Alfalfa	Plant	196	205	88.6	619	564
	Pulp	399	198	58.0	566	532
White clover	Plant	158	267	104	774	694
	Pulp	412	268	72.3	743	689
P-value	Fraction	<0.001	0.436	<0.001	0.046	0.214

Mean of three harvests (November 2013, June and September 2014) (Damborg et al., 2018)

Table 5.3 shows the changes in fibre fractions between the original biomass and the fibre pulp (Damborg et al., 2018). As expected the concentration of neutral detergent fibre (NDF), acid detergent fibre (ADF), cellulose and acid detergent lignin (ADL) in red clover, alfalfa, perennial ryegrass and white clover increased in the pulp compared to the original biomass. The fibre-associated CP was located in the hemicellulose, cellulose and lignin fractions indicating variable availability for ruminants. The same study evaluated the in situ digestibility, i.e., through nylon bag incubation in fistulated cows, and found a total tract digestibility that was lower in the pulp compared to the plant for all four plant species.

Table 5.3. Content of NDF, hemicellulose, ADF, cellulose and ADL in red clover and perennial ryegrass plant and pulp.

Plant Species	Fraction	aNDF (g/kg DM)	Hemi-cellu-lose (g/kg DM)	ADF (g/kg DM)	Cellulose (g/kg DM)	ADL (g/kg DM)
Red clover	Plant	413	147	266	213.7	52.3
	Pulp	589	210	379	297.2	81.8
Perennial ryegrass	Plant	503	254	249	235.3	13.7
	Pulp	694	353	341	307.6	33.4
Alfalfa	Plant	435	142	293	219	74.0
	Pulp	569	163	406	311	95.0
White clover	Plant	342	127	215	149.7	65.3
	Pulp	529	204	325	245.3	79.7
P-value	Fraction	<0.001		<0.001		0.003

Mean of three harvests (November 2013, June and September 2014) (Damborg et al., 2018). Hemicellulose content calculated as difference between aNDF and ADF. Cellulose content calculated as difference between ADF and ADL

Despite intended monogastric application, one experiment has dealt with evaluating the protein concentrate as a substitute for soybean meal for ruminants (Kragbæk Damborg et al, 2019) and Chowdhury et al., (2018) showed that it is easy to make rumen-protected protein out of grass protein concentrate, but also easy to overprotect.

5.2.2 Evaluation of feeding value

As part of the BioValue project, the feeding value of pulp was evaluated in dairy cows, where pulp silage was compared with grass-clover silage (Kragbæk Damborg et al., 2019). The experiment showed that the pulp ensiled well without additives and the cows had similar feed intake on DM basis regardless of silage type. Cows on pulp silage had a higher energy-corrected milk yield resulting in improved feed efficiency. Contrary to the in vitro and in situ digestibility analyses of the pulp by Damborg et al., (2018), the in vivo digestibility of CP and NDF was greater for pulp silage diets compared with grass-clover silage diets. This observation can most likely be explained by the physical processing of the pulp in the screw-press during biorefining, which disintegrates the fibres and increases the accessibility for the rumen microbes, thus increasing the degradability of the fibre and fibre-bound nutrients. The results imply that extraction of protein from grassland plants can increase the value of the fibre part of grassland plants. A new Finnish study investigates the effects of including pulp made from silage on feed intake, rumen fermentation, diet digestion and milk production in dairy cows but did not detect the same increased milk yield as the Danish study (Savonen et al., 2020). The pulp fractions can be ensiled well without any ensiling additives (Kragbæk Damborg et al., 2019; Larsen et al., 2019; Hansen et al., 2020). Larsen et al. (2019) recommend that the pulp fraction must be ensiled as soon as possible after processing of the grass biomass to ensure sufficient lactic acid production and quality of conservation.

An ongoing PhD project investigates the effect of harvest time and screw-press processing of grass on feed and protein value of pulp for dairy cows (Hansen et al, unpublished). Two different plant maturity stages combined with single or double screw press processing are being investigated. The preliminary results indicate that fibre digestibility increases with the number of screw-press processings for late harvest times. Furthermore, degradation of the protein remaining in the pulp was shifted from rumen degradation to small intestinal digestion resulting in improved protein quality (AAT value) of pulp compared to traditional grass silage for dairy cows.

6 Protein for food

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Protein from green biomass constitute a good source of protein for human nutrition. As stated in chapter 4.1 Biorefinery, the soluble protein fraction can easily be quenched out of the materials. In the fraction of soluble protein of leaves, the photosynthetic enzyme RuBisCO constitute by far the largest part (25-74% depending on the plant species) (Carmo-Silva et al., 2015). A size of 560 kDa makes it one of the largest enzymes in nature (Spreitzer & Salvucci, 2002). The structure of RuBisCO found in the green biomass is made of 8 large and 8 small subunits. RuBisCO on its own show an amino acid profile which in general meets the FAO recommendation for essential amino acids in human nutrition. It is lower in the sulphur containing amino acid, cysteine, but higher in the other sulphur containing amino acid, methionine. The monogastrics, rats and pigs are used as model for humans, please see chapter 5.1. Therefore, we are not addressing the nutritional value in this chapter. The chapter will cover protein fractionation for food, European Food Safety Authority (EFSA) approval, toxic and anti-nutritional factors (ANFs), allergenicity, and functional properties. In this chapter we decided to review more than what is found in Denmark as less is published and no commercial production of food quality protein from green biomass is implemented yet. Parts of this chapter build on a newly published review on biorefinery of green biomass for food (Møller et al., 2021, in press).

6.1 Protein fractionation for production of food protein products – removal of green colour

The protein extracted from green biomass can be precipitated directly from the green juice without further purification. However, this protein concentrate will be green due to the content of chlorophyll in the plants and the protein concentration in the concentrate will normally be around 40-60% of DM (Amer et al., 2021; la Cour et al, 2019; Nissen et al., 2021; Stødkilde et al., 2019 and chapter 5). This protein may be used in food products, but the use will be limited not only by the colour but also by a grassy flavour and odour of the green protein concentrates (personal communication, industrial partners).

It is possible to remove the green colour and grassy odour by purifying the proteins. The protein is fractionated into a green fraction containing proteins with low aqueous solubility and chlorophyll, and a white protein fraction containing the more water soluble proteins. The protein concentrates from the white protein fraction will have a higher protein content in DM, higher aqueous solubility and likely better functional properties when compared to the unpurified green protein.

A number of different methods have been tested for the production of white protein concentrate. The method that has been most commonly used is heat fractionation. In this method, the green juice is heated to 40-60 °C for a short period of time. This will lead to precipitation of the green protein fraction (Damborg et al., 2020; Edwards et al., 1975; Lamsal et al., 2003) and the proteins left in the juice after precipitation of the green protein fraction will then be the white protein fraction. The heat fractionation is depending on temperature, holding times, pH and salt and even small changes in the different parameters can affect the yield of white protein (de Fremery et al., 1973). Addition of divalent salts have been suggested to increase the precipitation of the green protein fraction (De Jong et al., 2015; Gwiazda & Saio, 1981). Very recently, Nynäs, Newson, & Johansson (2021) investigated the effect of temperature on precipitation of the green protein fraction for 9 different leafy plant types and found that the optimal temperature for green protein precipitation depends on the plant type. In the classical heat fractionation method, the white protein part is concentrated using heat denaturation at 80-90 °C (Edwards et al., 1975). However, since the high temperature heat denaturation produces protein concentrates of low solubility (Nissen et al., 2021), other alternative methods for concentration of the white protein fraction have been tested such as ultrafiltration (Knuckles et al., 1975; Knuckles et al., 1980a; Lamsal & Koegel, 2005; Martin et al., 2019), acid precipitation (Miller et al., 1975) or ion exchange chromatography (Martin et al., 2014). Additionally, using heat fractionation to obtain white protein may compromise the protein quality and solubility.

Microfiltration has also been used to remove the green colour (Eakin et al., 1978), but this filtration could only remove 90% or the green colour. Adding flocculants to the green juice can improve the separation in microfiltration (Knuckles et al., 1980b), but the method has not been further investigated. Another method uses the fact that the green protein fraction precipitates to a higher degree than the white protein fraction when lowering the pH (Merodio et al., 1983; Merodio & Sabater, 1988; Satake et al., 1985). The acidification can be achieved by addition of acid, but it has also been suggested to use lactic acid forming bacteria for the acidification (Lamsal et al., 2003). Fractionation by acidification have not been tested in larger scale.

Instead of fractionating the protein, it has also been suggested to remove chlorophyll from the protein using organic solvents. Chlorophyll is hydrophobic and can be extracted from the protein while simultaneously precipitating the protein fraction by addition of acetone or isopropanol (Bray & Humphries, 1978; Bray et al., 1978; Hove & Bailey, 1975; Satake et al., 1984). However, this use of organic solvents are nor desirable when aiming at production of food grade protein and proteins denatured by organic solvent is expected to have low solubility in water. Alternatively, a method for chlorophyll removal using activated carbon has been described in a patent (Van De Velde et al., 2011).

Since current, only large scale production biorefineries focus on production of feed protein in Denmark (chapter 5), these production plants do not fractionate the protein. However, there is a demonstration scale plant in The Netherlands, which focuses on production of purified RuBisCO from sugar beet leaves (www.greenproteinproject.eu). The exact process is not available.

6.2 European Food Safety Authority approval

Protein concentrate from alfalfa is the only leaf protein concentrate from leafy green plants that has been approved for human consumption by the EFSA (Bresson et al., 2009). The approval was granted in 2009. However, the recommended daily dose is relatively low at 10 g concentrate/day, due to the possible presence of anti-nutritional factors (ANFs) in the alfalfa protein concentrates, where saponins, phytates, and L-canavanine, and secondary metabolites such as phytoestrogens (coumestrol and isoflavones) but also β-carotene were specifically mentioned by EFSA. Besides the phytoestrogens, the leafy green plants also contain other polyphenols that can affect the nutritional value of the extracted protein.

6.2.1 Toxic and anti-nutritional factors in plants

Anti-nutritional factors may impair the absorption and utilization of other nutrients. For example, protease inhibitors, tannins, saponins, and lectins affect the protein utilization and lower the protein digestion, while some others e.g. phytate affects the absorption of micronutrients as minerals and vitamins (Makkar et al., 1993). The plants used for extraction of protein will also contain some of these although in varying amounts. In this chapter, the ANFs found in plants that potentially can be used for green protein production will be reviewed. The main leafy crops that are considered for production of food protein in Denmark are alfalfa, red clover, white clover and grasses and this report will focus on the content of ANFs in these plant species. Besides these, other leafy plants species can potentially be considered for production of protein, but the potential contents of ANF in these species will not be considered here. Since only alfalfa has been previously used for production of protein concentrate, this is also the plant species that has been analyzed the most for the content of ANFs. The biorefinery process will likely affect the levels of the different anti-nutritional factors in the final protein products, but it needs to be further investigated.

6.2.2 Phytochemicals

Phytochemicals is a very broad category of compounds formed in plants by the plant metabolism with the ability to affect human health. Phytochemicals are traditionally divided into two major groups, carotenoids; including carotenes and xanthophylls being pre-cursor of vitamin A and providing colour in the yellow-dark orange area, and polyphenols; including phenolic acids, flavonoids, tannins, and stilbenes/lignans (Heneman & Zidenberg-Cherr, 2008). Besides these, groups of N-compounds, organosulfur compounds, carbohydrates, and lipids are also categorized as phytochemicals. The phytochemicals can either be anti-nutritional or even toxic but may also have positive effects on human health. Although not with solid evidence, some phytochemicals are suggested to protect against various diseases. Polyphenols are considered to reduce the risk of e.g. cardiovascular disease, cancer, and diabetes (Del Rio et al., 2013) but many inconsistent data are present in literature. Therefore, no definitive recommendations for the use of these compounds in the prevention of cardiovascular disease and cognitive decline can be given (Potì et al., 2019). Lignin-amide compounds e.g. display in vitro anti-inflammatory activity (Sun et al., 2014).

Phytochemicals are often concentrated in the outer cell layers of the seeds of e.g. soy beans, pea and faba beans and hulling processes often affect their content (Mattila et al., 2018). For leafy green biomass this processing procedure is not an option and therefore the phytochemicals constitute a significant implication in the extraction and purification of proteins from these materials as certain phytochemicals may possess anti-nutritional or toxic properties, at certain doses to humans and animals.

6.2.3 Saponins

Saponins are amphipathic glycosides, which are grouped structurally by having one or more hydrophilic glycoside moieties combined with a lipophilic triterpene or steroid derivative. They can be divided into groups based on the soap-like foam they produce when shaken in aqueous solutions. The saponins have a bitter taste, which is a limiting factor on the intake of saponin-rich protein products. In addition to this, saponins may affect the gastrointestinal lining, contributing to leaky gut syndrome and autoimmune disorders. The saponins are particularly resistant to degradation in the human digestive system and have the ability to enter the bloodstream and trigger immune responses (Sen et al., 1998). The saponins, soyasapogenol B, hederagenin, bayogenin, medicagenic acid, lucernic acid, and zanhic acid are the main anti-nutritional compounds in alfalfa leaves (Sen et al., 1998); they have traditionally been regarded as limiting factors for its usage as animal feed. However, other studies have shown beneficial effects of saponins, e.g. as cholesterol lowering (Vinarova et al., 2015). Today alfalfa is used widely as feed for both ruminants and monogastric animals mainly as silage but also as meal (Liebhardt et al., 2019; Sinclair et al., 2015). When considering the higher levels present in other food products, EFSA has indicated that the level of saponins in alfalfa powders did not raise a concern at a recommended daily intake of 10 g alfalfa protein concentrate (Bresson et al., 2009). However, for higher recommended daily intake and for different processing it should be considered for future approval.

6.2.4 Polyphenols and polyphenol oxidase

Phenolic compounds are a large group of plant metabolites with more than 6000 different identified compounds (Kroll et al., 2003). The presence of polyphenols has been shown to lower the nutritional value of leaf protein (Rambourg & Monties, 1983), but the polyphenols may also affect protein quality through various oxidation mechanisms. Present in the leaf chloroplasts is the polyphenol oxidase (PPO) enzyme (Boeckx et al., 2015), which is normally separated from the polyphenols in different compartments of the cell. However, during processing and lysis of the plant material, the PPO will come in contact with the polyphenols allowing PPO to catalyze the oxidation of polyphenols to produce quinones. The formed quinones are highly reactive compounds that can non-enzymatically react with themselves to form brown polymeric pigments (Bittner, 2006) or with amino acids in either free form, in peptides and when present in proteins (Bittner, 2006; Pierpoint, 1966, 1969a, 1969b). More specifically, quinones can react with lysine, cysteine, methionine, and tryptophan in proteins (Hurrell et al., 1982; Kroll et al., 2003). The reaction between polyphenols and proteins in model systems changes the physicochemical properties of the protein including protein dimerization through cross-linking and reduced solubility (Amer et al., 2021; J. Kroll & Rawel, 2001; Kroll et al., 2000; Rawel et al., 2000) and lower the in vitro protein digestibility (Amer et al., 2021; Kroll & Rawel, 2001; Kroll et al., 2000), and the nutritional value of the protein in rats (Hurrell et al., 1982; Matheis & Whitaker, 1984). However, a recent study found that although sulphite addition increased the level of native rubisco from alfalfa, the solubility of the protein produced by acid precipitation was actually reduced by the sulphite addition (Tanambell, Møller, Corredig, & Dalsgaard, 2022). Different plant species are known to have widely different PPO activities. Red clover has a high PPO activity (Jones et al., 1995) although red clover species with lower PPO activity are known (Lee et al., 2004) and alfalfa has low PPO activity (Sullivan & Hatfield, 2006).

PPO activity and enzymatic browning during processing and inhibition of the enzyme can be prevented by physically treatment (e.g. heat, hydrostatic pressure treatment, gamma radiation and pulse electric field) or by addition of antioxidants (Queiroz et al., 2008). Steam blanching of alfalfa whole plant followed by drying and alkaline protein extraction reduced both PPO and peroxidase activity in the protein extracts (Hadidi et al., 2019). Own data has however revealed that pre-heat treatment makes it difficult to extract the protein afterwards. Ascorbic acid most likely prevents oxidation by reducing the quinones formed by PPO (Narváez-Cuenca et al., 2011; Pierpoint, 1966). However, ascorbic acid is oxidized and will be consumed by continuous quinone formation (Pierpoint, 1966; Özoğlu & Bayındırlı, 2002). Other antioxidants are the sulphur containing such as metabisulfite, sulphite and cysteine that react with the quinone forming sulfobenzoyl derivatives of the polyphenolic compounds (Embs & Markakis, 1965; Narváez-Cuenca et al., 2011). Metabisulfite or sulphite have been widely used to prevent browning reactions during the production of leaf protein (Edwards et al., 1975; Fiorentini & Galoppini, 1981; Martin et al., 2019; Sheen, 1991). However, the use of sulphite in food production has been associated with some adverse health effects, so it is important to verify if sulphite follows the protein in the biorefinery procedures. Removing the polyphenols from the leaf juice (i.e. extraction with organic solvent or adsorbent resins (D'Alvise et al., 2000; Firdaous et al., 2017)) is another method that has been considered to prevent enzymatic browning and thereby secure the protein quality. However, this method has not been tested in larger scale and practically it may be difficult to remove the polyphenols before they are oxidized.

6.2.5 Tannins

Tannins are astringent polyphenols that bind proteins, amino acids and alkaloids, leading to precipitation hereof. Tannins is high in tea, so many people are already consuming a lot of tannins on a daily day basis. Tannins interaction with protein and amino acids may affect human health (Chung et al., 1998). The tannin content in green leaf vegetables of 13 different plants has been reported, and ranged between 0.61 and 2.05 mg/g with the exception of *Coleus aromaticus* (0.15 mg/g) and *Delonix elata* (13.3 mg/g) (Gupta et al., 2005).

6.2.6 Phytoestrogens – Isoflavonoids, coumestans and lignans

Phytoestrogens, typically polycyclic phenols, are secondary plant metabolites having a weak estrogen effect. Phyto-estrogens are common in many plants, including soy. The main isoflavones are genistein, daidzein, glycitein, formononetin, biochanin A and puerarin, and they possess estrogenic properties (Aguilar et al., 2015). Some of these compounds are found in red clover, alfalfa and grasses (Aguilar et al., 2015). Formononetin, biochanin A, daidzein, and genistein are found in clover, and coumestrol found in alfalfa. The phytoestrogen content in red clover depends on variety and season (Johansen et al., 2020). They are able to bind to the estrogen receptor and can potentially result in harmful changes in hormone levels and are hence considered endocrine disruptors, i.e. plant-derived compounds with estrogenic activity. How much phytoestrogen is present in different protein products have not been investigated and needs further attention.

6.2.7 Non-proteinogenic-amino acids

Many of the 200 or so non-protein amino acids synthesized by higher plants are related structurally to the constituents of common proteins. The toxic L-canavanine is a non-protein amino acid and an arginine analog that was specifically addressed by EFSA. Production of canavanine-containing proteins can disrupt critical reactions of RNA and DNA metabolism and protein synthesis. Canavanine also affects the arginine metabolism and uptake (Rosenthal, 1977). L-canavanine has been investigated in a Polish study that found a 110 µg/g DM in alfalfa juice and 4.5 µg/g DM in protein-xanthaphyll extract (Gaweł, 2012).

6.2.8 Phytic Acid / Phytate

Phytate was one of the ANFs specifically addressed by EFSA in the alfalfa approval. Phytate is the six-fold dihydrogenphosphate ester of inositol. At physiological pH, phytate is partially ionized at the phosphates resulting in the phytate anion. This is a colourless species that has a significant role in plant nutrition as storage form of phosphorus in plant tissues, e.g. seeds and bran (Schlemmer et al., 2009). It is also found in grains, cereals and legumes and interferes with the absorption of minerals. Phytic acid phosphorus was found to represent from 10 to 15% of total root and crown phosphorus in alfalfa (Campbell et al., 1991). However, the phytic acid was much lower in alfalfa than in soy and grain (Eeckhout & De Paepe, 1994) and in the alfalfa powder accepted for a daily intake of 10 g in 2009 EFSA also stated that it was lower than what is seen in other plant foods, thus not constituting a problem for alfalfa. The phytic acid and phytate have strong binding affinities to calcium, iron and zinc, whereby their absorption is inhibited. However, a high fraction of phytate (up to 2/3) can be degraded in the stomach and small intestine, when the diet contains intact phytase, an enzyme that degrades phytate, otherwise the degradation of phytate in the upper part of the gut decreases to 0-28% (Schlemmer et al., 2009). It is not clear whether or not this will cause problems in protein extracts of alfalfa or green biomass in general.

6.2.9 Oxalic acid / Oxalate

Oxalic acid is a dicarboxylic acid present in many plants, which forms calciumoxalate with calcium resulting in low solubility. Foods which contain large concentrations of oxalic acid, e.g. the green leaves of spinach (~9-10 mg/g) and in the stems of rhubarb (~4 mg/g) (Kennedy & Durfee, 2011), can reduce the absorption of calcium in the gut. Hay of alfalfa holds 5-8.7 mg/g DM (Hintz et al., 1984). A common way to overcome this is the addition of calcium chloride to such foods, which causes precipitates in the form of calciumoxalate salts, thereby increasing the amount of free calcium and improving calcium absorption. Similar to tannins, oxalates are, besides leaves of spinach, found in the highest quantities in sesame seeds, soybeans, and black and brown varieties of millet. The content of oxalic acid in the protein fraction of green leaf plants for feed and food is important for managing the mineral absorption.

6.2.10 Lectins

Legume lectins are a group of glycoproteins found mostly in seeds (Loris et al., 1998). Only few reports exist on the presence of lectins in green-leaf plants, although gene sequences for lectins have been found in both red and white clover (Gubaidullin et al., 2007), and clusters for agglutinin gene sequences have been found in perennial ryegrass (Lolium perenne L.) and meadow fescue (Festuca pratensis Huds.) (Tamura & Yonemaru, 2010). No studies relate lectin constituents of these plants to food and their potential consequences for human digestion.

6.3 Allergenicity

Allergenicity is defined as immune-mediated adverse reaction to foods, which causes different clinical sign and symptoms. There are anaphylactic reactions with a reaction between a specific food and the immunoglobulin IgE, and a non IgE-mediated food allergy where other parts of the immune system reacts.

The IgE-reaction is caused by a reaction between specific proteins and the immunoglobulin, known as an antigen-antibody reaction. It relies on a recognition and binding of a specific amino acid sequence or patches in a food protein by the immunoglobulin. Hence, allergenicity may be investigated from sequence similarity with known allergens (EFSA Panel on Dietetic Products & Allergies, 2014) also known as the *in silico* approach. Different criteria have been suggested to count for a protein to be characterized as an allergen. However, FAO/WHO/EFSA gave the following criterion; over a sliding window of 80 residues a value of 35% amino acid identity with known allergens is the used criteria for prediction of the allergenic risk of a new protein (Ladics, 2019). However, the recognition of the immunoglobulin can also be caused by the folding of the protein, where the 3D protein structure comes into play. However, immunological and clinical data is also needed to classify a food protein as an allergen.

The clinical tests used today is the skin prick test and a measurement of serum IgE levels. Quite some animal protein are recognized as allergens, but only few studies with RuBisCO and alfalfa protein are found in literature. RuBisCO on its own is recognized as a protein with relatively low allergenicity (Smit et al., 2016) compared to other protein sources, namely soy. In a study with participants allergic to soy, immunoblotting showed that IgE binds to soy protein extract but not to RuBisCO. However, using the ELISA technique shows some binding for some patients (Hoff et al., 2007), thus being less persuasive. A single study with only one participant responding to RuBisCO from spinach and tomato (Foti et al., 2012) indicated that RuBisCO may give problems for some people. Alfalfa protein was recently investigated by an *in silico* approach and three different allergen families namely lipid transfer, thaumatin-like, and Bet v 1-like protein families were suggested to be potential allergens in alfalfa (Yakhlef et al., 2020). However, more studies performed as *in vitro* or *in vivo*, and subsequently clinical evidence, are needed to verify these results.

6.4 Functionality - leaf protein in food applications

Not only the nutritional value and ANF are important when considering the use of protein from green biomass for food application. The functional properties are equally important and often more important to fulfil the needs in different food applications. In general, the overall functional properties of proteins as food ingredients are highly dependent on the processing conditions (Corredig et al., 2020). Furthermore, physical and chemical food relevant conditions of pH, salt content, temperature, and concentration are all parameters that affects the functional properties; protein solubility gelation, foaming, emulsification and water holding capacity. RuBisCO, the major photosynthetic protein in green biomass, is highly important for the functional properties of protein extract and isolate, as it constitute 50-60% of the soluble proteins.

6.4.1 Solubility of proteins

In most food applications where protein acts as a functional ingredient, protein solubility in water is essential. However, there are cases such as emulsions and foams where the interfacial properties do not necessarily require high solubility of the protein but rather the amphiphilic nature of the protein is highly important.

The solubility of proteins extracted from alfalfa depends strongly on the pH of the system (Nissen et al., 2021) and the processing (Nissen et al., 2021). The isoelectric point of RuBisCO determine the solubility of the protein extracts. Hence, RuBisCO having an isoelectric point around 4.5 results in a minimum solubility at pH 3.5-5 (~10% soluble) (Bahr et al., 1977). From pH 5 the protein solubility increases linearly to a maximum of ~80% at pH 10 (Knuckles & Kohler, 1982; Lamsal et al., 2007; Martin et al., 2019; Wang & Kinsella, 1976b). In alfalfa protein extracts, increasing pH to 11 and 12 and readjusting to pH 7 increases the protein solubility at pH 7, but the alkaline pH also induce formation of protein crosslinking products, lanthionine and lysinoalanine (Nissen et al., 2021).

The method used for protein extraction also affects the solubility. Even though alkaline treatment of protein extracts increases the solubility as shown in Nissen et al. (2021), alkaline extraction limits the solubility of protein from alfalfa leaves (Hojilla-Evangelista et al., 2017). The method used for precipitating the protein also highly affect the solubility. Alfalfa protein isolate shows higher solubility when extracted by ultrasound-ultrafiltration-assisted alkaline isoelectric precipitation than those extracted by heat or alkaline isoelectric focusing precipitation (Hadidi et al., 2020). Leaf white protein precipitates produced by heat denaturing at 80 °C have a very low or negligible solubility, whereas use of acid precipitation results in higher solubility (Betschart, 1974). Low temperature further increase the solubility of the protein (Miller et al., 1975). Alternatively, a white protein product with good solubility can be produced by concentrating using membrane filtration (Knuckles & Kohler, 1982; Lamsal et al., 2007). Drying conditions also affects the protein solubility. For white alfalfa protein, increased outlet temperature, from T = 85 °C to T = 140 °C, during spray-drying inversely affect the solubility (Knuckles & Kohler, 1982). For spinflash and vacuum drying, protein extracts, vacuum dried protein has the highest solubility (Nissen et al., 2021). No matter if it is precipitation or drying temperature that exceeds the denaturation temperature, which Lamsal et al. (2007) determined to Td ~ 70-75 °C, it seems, not unexpectedly to decrease the protein solubility. Upon denaturation, the protein molecular structure undergoes conformational changes that results in higher surface hydrophobicity, which has a significant effect on the solubility in an aqueous solutions.

6.4.2 Foaming properties

Due to their amphiphilic molecular structure, proteins are surface active due to the presence of both hydrophilic and hydrophobic regions on their surface. The foaming capacity depends on the surface activity of proteins, which determines their suitability for preparing aerated foods, such as bakery, confectionary, and beverages. Foaming capacity and stability depends on the unfolding of protein upon mechanical stress. Upon unfolding, the hydrophobic and hydrophilic regions orientate to an air-water interface, and the formation of a stabilizing protein film surrounding the foam bubbles (Hammershoj et al., 1999). Foaming is concentration dependent and up to a critical concentration, where a saturation level or steady state condition is reached, increased protein concentration increases the foaming capacity. After reaching the critical protein concentration, incorporation of more protein at the interface is no longer possible at the monolayer of the air-water interface (Hunter et al., 1990).

The protein extraction method also affects the foaming properties. Proteins obtained from NaCl, NaOH, and TRIS-buffer extraction of alfalfa leaf shows lower foaming properties compared to protein obtained by pressing. The foaming capacity was also shown pH dependent with lowest capacity around pI (Wang & Kinsella, 1976a).

At pH close to is isoelectric point (pH 4), RuBisCO isolated from sugar beet leaves exhibits a foam overrun of 85-100%, which is significantly higher than whey and soy protein isolate. At pH 4, there was a need for at least 5 g/kg protein to reach a foam overrun capacity resembling whey and soy protein isolate with an overrun capacity greater than 60%. Foam stability of RuBisCO protein was 3 and 6 times higher than soy protein and whey protein, respectively.

Alfalfa leaf protein has higher foam capacity, but results in a less stable foam than of ovalbumin, the latter being the dominant protein in egg white (Relkin et al, 1999). Another study showed that whipped alfalfa leaf protein concentrate performed equally with respect to foaming capacity compared to egg white protein but within 2 h, the alfalfa leaf protein retained higher foam stability than the egg white protein (Knuckles & Kohler, 1982).

Even though using a non-food grade extraction solvent (hexane) for defatting, Hojilla-Evangelista et al. (2017) investigated the foaming capacity at different pH values. At concentration of 10 g/L the bubbled foam capacity, the highest foam capacity and stability were obtained at pH 2 compared to pH 7, and pH 10, where foam volumes were low with little stability, showing immediate collapse. At pH 2 the foam properties of alfalfa protein equals those of soy protein concentrate (Hojilla-Evangelista et al., 2017). Knuckles and Kohler (1982) also reported that a whipped foam of alfalfa leaf protein performs highest capacity at lower pH values (pH 3-6) with the highest stability at pH 4.5. Around the isoelectric point, less repulsion is expected than above the isoelectric point of the protein. Together with a low electrostatic repulsion at the isoelectric point it facilities the adhesion of proteins at the air-water interface, hence creating a stable foam.

6.4.3 Emulsifying properties

An emulsion consists of at least two immiscible liquid phases, e.g. oil dispersed in the water phase and stabilized by an emulsifying agent, e.g. proteins. Many proteins are known to be emulsifying agents, including plant proteins from lentils (Can Karaca et al., 2011) and potatoes (Schmidt et al., 2018). RuBisCO is suggested to be a protein of relevance for food emulsions with performance comparable to soy and egg white proteins (Barbeau & Kinsella, 1988), or even superior to egg white proteins (Lamsal et al., 2007).

Emulsions of alfalfa protein defatted by acetone extraction have been studied as function of concentration, and salt and sucrose addition (Wang & Kinsella, 1976b). These authors observed the highest emulsifying capacity of alfalfa leaf protein at pH 5 and much lower at both higher and lower pH values (Wang & Kinsella, 1976b). In contrast, both the Emulsifying Activity Index and the Emulsifying Stability Index of alfalfa leaf protein extracted under alkaline condition from alfalfa leaves h increase with pH (pH 2, 7, and 10) (Hojilla-Evangelista et al., 2017). This indicates improved emulsifying properties of alfalfa leaf protein at alkaline pH, where unfolding of the proteins increases the surface hydrophobicity of the protein when hydrophobic amino acid side-chains become exposed at the surface allowing for interactions at the oil-water interface (Hojilla-Evangelista et al., 2017).

RuBisCO obtained from sugar beet leaves showed larger mean diameter than those of whey protein isolate but smaller than those of soy protein isolate (Martin et al., 2019). At high protein concentration (10 g/kg) the emulsion droplet diameters of RuBisCO are more comparable to whey protein than at protein concentration < 5 g/kg, but more stable RuBisCO emulsion is seen pH 4 than at pH 7. At present no study show emulsifying properties of grass or clover proteins.

6.4.4 Gelation properties and gel texture

A protein gel is a network of protein molecules in an aqueous solution where the protein molecules aggregate and/or bind together to form a network. Hence, the water or aqueous solution is bound in the gel network resulting in a macroscopically semi-solid structure. Gelation is often induced by a physical treatment, either thermal treatment or shift in pH (Martin et al., 2019). Gelation is also affected by the salt concentration.

Heat treatment around the denaturation temperature (72 °C, 30 min) of alfalfa leaf protein expose the hydrophobicity of the protein surface resulting in gel formation at low concentrations (1-2% protein) (Knuckles & Kohler, 1982).

A 5% alfalfa leaf protein gives a gel strength twice as high as of a 15% soy protein isolate (Knuckles & Kohler, 1982). RuBisCO protein from sugar beet leaves forms stronger gels at low concentration compared to whey protein and soy protein isolate (Martin et al., 2019).

Soluble alfalfa protein shows significant cold-setting behaviour and the aggregates are suggested to be either branched or clustered with a low density and shear thinning behaviour after heating at 90 °C for 1 h followed by cooling (Lamsal et al., 2005). The same group compared a solution of 7% soluble alfalfa leaf protein and 13% whey protein isolate at pH 7. Both type of protein solutions form standing gels, although the gels formed are different types (Lamsal et al., 2007).

Another study compared 2.5-10% protein concentration of RuBisCO from spinach with whey and egg white protein in combination with and salt levels of 0-0.2 M NaCl. The spinach RuBisCO gels shows a lower onset temperature and higher storage modulus (G') and gel strength in texture analysis compared to gels of whey protein and egg white protein (Martin et al., 2014). The density, also referred to as microstructure, of the RuBisCO gel is correlated to the protein concentration. The RuBisCO gels are more affected by ionic strength than the egg white and whey protein gels. This is caused by the RuBisCO protein structure, which is highly dependent on the RuBisCO subunit being held together in the gel by electrostatic interaction. Hence, addition of NaCl to the gel system at 5% protein resulted in lower gel strength while NaCl addition to a 10% protein concentration do not affect the gel microstructure (Martin et al., 2014). Most recently, Nissen et al. shows remarkable gelating potential of alfalfa protein concentrate with the alkaline pH shift method (pH 11) with re-adjusting to pH 7, reaching 2584 Pa with 72 g/L protein (Nissen et al., 2021).

6.5 Conclusion on food properties of protein from green biorefining

In conclusion, RuBisCO and alfalfa protein show promising functional properties, making it a potential substitute for animal protein ingredients. Thus far, when considering leaf protein for food the focus has been on proteins from alfalfa and sugar beet leaves. However, the RuBisCO protein is very preserved among different plant species in terms of protein sequence and structure, why RuBisCO obtained from other leafy plants, such as grasses and clovers, may have similar functional properties. At the same time RuBisCO shows relatively low allergenicity, so a purified RuBisCO product may serve as a potential source for highly challenged multiallergenic population. Other proteins but RuBisCO present in different green biomass may have allergenic potential but more investigations are needed to resolve this. Today, alfalfa protein is approved in food applications, but only based on a limited daily intake. Still there is a way to go concerning description of the full matrix both for alfalfa and other green biomass. Different antinutritional factors are present in different plant species and they need to be quantified in each specific case of processing as they may concentrate in the protein concentrate depending on the specific processing used to produce the protein. Hence, any new protein product produced from either alfalfa, clover or grass needs a new EFSA approval before the protein can be used in food products, but if we want to meet a 70% reduction in carbon emissions, plant protein from alternative sources like green biomass traditionally used for feed will need attention for food applications.

7 Perspectives in organic farming

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7.1 The challenges

In organic farming it is difficult to meet the need of proteins with the correct amino acid profile for pigs and poultry since in organic farming no use of synthetic amino acids are allowed to balance the diet. This increases the risk of oversupplying pigs and poultry with protein to meet the need of the individual essential amino acids. An oversupply of protein is likely a contributing factor to the lower feed conversion of organic poultry and pig production compared to conventional production. This increases the environmental footprints of the organic production e.g. exemplified in a considerable higher ammonia emission from the manure than in conventional production (Hermansen et al., 2015).

Regionally produced and 100% organic feed is an important goal in the organic livestock production. However, in the EU project 'Improved contribution of local feed to support 100% organic feed supply to pigs and poultry' (ICOPP), it was calculated that the self-sufficiency (considered at the EU level) with organic proteins to monogastric livestock was low - 50% for lysine and 40% for methionine (Smith et al., 2014). An increasing organic livestock production at EU level continues to put pressure on the supply of organic protein sources suitable for livestock feeding.

The above challenges have repeatedly postponed a transition to 100% organically produced feed components for organic pigs and poultry in the EU. Until Jan 01 2022, pigs and poultry may be supplied with up to 5% non-organic feed (protein-sources) in order to better meet their nutrient needs (EC, 2021). According to an industry agreement within the Danish organic pork sector, pigs must be fed with 100% organic feed ingredients. However, due to a reduced import of soya from outside Europe – partly caused by the Covid19 pandemic – a temporal dispensation (until 01 Jan 2022) has recently been implemented (Holdensen, pers. comm. 2021) accentuating the need for locally produced protein sources.

A number of feed materials can be used to fully or partially meet livestock amino acid needs, e.g. seeds of esparcette or 'grass seed pea', processed sunflower cakes where the protein is concentrated, starfish and mussel meal or meal from insects. A common feature of these solutions is that the feed material is expensive due to low yields (esparcette and grass seed pea), cost-effective technology is not fully developed (insect meal) or the availability is limited (starfish meal) (Smith et al., 2014; Steenfeldt & Poulsen, 2018; Studnitz, 2019). In the above-mentioned ICOPP project, it was concluded that green legumes like alfalfa were the most promising in terms of providing the necessary organically produced protein to meet the needs of pigs and poultry, because they are crops, which can produce high yields even under organic production. In addition, they are crops that fit well into organic crop rotation, and do not require synthetic nitrogen fertilizer.

Use of whole green mass as feed (with the objective of supplying the animals with protein) results, however, in a lower feed conversion since the monogastric livestock cannot utilize the fibre part very well (Smith et al., 2014).

Thus, the biorefinery technology seems to represent a promising pathway to produce protein for organic monogastrics production. The potential of “green protein” in organic livestock feeding has been discussed in Steenfeldt and Poulsen (2018) and evaluated in a number of recent national projects OrganoFinery, Multiplant, SuperGrassPork, and Green-eggs. A bio-refinery was established on a commercial farm in 2020 to produce protein from organic grass as part of the national project TailorGrass. The produced green protein has been applied in several commercial organic feed mixtures for pigs and the effects on animal performance and health are currently evaluated.

7.2 Example of industry perspectives in organic livestock production

The organically managed land in Denmark amounts in year 2019 to 301,000 ha – in latest year showing an increasing trend. Of these approx. 160,000 ha are located on dairy farms or support roughage production and 30,000 ha on farms for horticulture and specialised plant production. The remaining area (110,000 ha) is used for mixed farming, including suckler cows as well as pig and poultry farming (Landbrugsstyrelsen, 2020).

The largest proportion of land is used for grass/clover grass/other green fodder (131,000 ha), while approx. 100,000 ha is used for cereal production. Thus, contrary to the situation in conventional farms, organic farms have much more grass-clover in the crop rotation (to support the supply of nitrogen through biological N-fixation) and less cereal.

A typical organic dairy farm has around 55% grass-clover, 20% cereals and 20% whole crop silage or maize silage in the rotation (Kristensen et al., 2020), and grass-clover constitutes the main silage type used during winter. The high proportion of grass-clover in the rotation facilitates a high intake of fresh grass through grazing during summer (40% of annual grass production), but at the same time makes much grassclover available for conservation due to the high growth in early summer.

Based on these numbers it could be considered to use half of the grass-clover produced for silage on organic dairy farms for biorefining corresponding to a theoretical area of 26.000 ha. (160,000 ha at dairy farms of which 55% with grass-clover, 60% of this for silage and 50% of this for biorefining).

The organic farms, besides dairy, has on average 20% of grassland in the rotation, some of the area for grazing and outdoor access for livestock, but also some areas for silage and green manure (Kristensen et al., 2012). Therefore, it is probably not feasible in general to reduce this proportion too much further. However, like for dairy farms it could be an option to use part of the grassland on these farms for biorefining purposes. Using 1/3 of the grassland at these farm for biorefining equals 14,000 ha (131,000 organic grassland – dairy grassland (160,000*0.55=88,000) = 43,000 * 0.3 = 14,000 ha).

Assuming that in total 40,000 ha of organically managed grassland could be used for biorefining, with a production annually of 7,000 kg DM/ha, 19% of DM in a protein fraction with 42% protein of DM equaling 559 kg protein per ha, or in total from 40,000 ha 22,000 tonnes of protein could be achieved.

Assuming Danish organic pig and poultry production includes 12,000 sows with 240,000 finishers and 1,200,000 hens (Landbrugsstyrelsen, 2020) producing 23m kg eggs per year, the need for protein in total can be estimated to 23,000 tonnes protein. If 40% of this could be from biorefining of grass-clover it equals 9,500 tonnes protein or 15,000 ha organic grassland.

Thus there would be room for an export of another 12,500 tonnes of protein. As previously mentioned, there is a shortage of protein feed for monogastrics in the EU generally, so one can assume good market opportunities. The fibre fraction containing 58% of the DM, with17% protein in DM, can be used as roughage for dairy and other livestock, but if biorefinery protein is exported there will be a need for alternative protein and energy sources, primarily to dairy livestock feeding, if production should be maintained.

8 Economic assessment of a small scale green biorefinery

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8.1 Introduction

As stated in chapter 4, Green Biorefining (GB) is a fundamental concept that "represents the sustainable processing of green biomass into a spectrum of marketable products and energy" (McEniry and O'Kiely, 2014). GB can be seen as a technology platform that integrates a variety of different sustainable solutions in order to produce a variety from food and feed to biomaterials, biofuels and bioenergy based on multi-product cascading. As described above in chapter 4, a green biorefinery will fit in various production or value chain schemes and configurations. As a basis for the following business economic assessments, it has been decided to build on technical data from a basic decentralized stand-alone biorefinery plant producing soy quality green protein, fibre pulp and brown juice.

8.2 Short technical description

The production of green protein from grass-clover is not yet fully commercialized in DK, and therefore we have a lack of full-scale experience for the biorefinery concept. As stated in chapter 4, currently there is a medium scale pilot plant at AU Foulum and a smaller scale pilot plant at The Danish Technological Institute. Two semi-commercial farm-scale plants have been built for the season 2021 based on the experiences from the mentioned pilot scale plants and various demo scale projects. The capacity of the plants is about 20,000 tonnes of DM grass-clover input, annually (Morten Ambye-Jensen, pers. comm., 2021).

A similar size decentralized biorefinery plant with capacity of 20,000 tonnes of DM grass-clover input and an output of 3,600 tonnes DM protein, 14,000 tonnes of fibre pulp DM and 2,500 tonnes DM brown juice has been described and used for economic assessment in Jensen and Gylling (2018) and Børgesen et al. (2018). The size and capacity is chosen based on the experiences from the pilot scale and field scale demo activities. The necessary farming area to supply the grass-clover is estimated to equal an area of 2,600 hectares. The assessment is made based on three price-levels, conventional, non-GM and organic protein products.

8.3 Organization in practice

Based on the experience from the green drying industry, it is assumed that harvest and logistics/transport to the biorefinery is managed centrally by the biorefinery or hired contractors. The farmer grows the grass-clover and sells it to the biorefinery as a standing crop, and the biorefinery manages the harvest and transport in terms of scheduling and operations planning.

Pilot scale experiences have shown that an efficient logistics setup is extremely important for the operations efficiency and quality of the harvested grass-clover and thus for the processing at the biorefinery plant and the products from the biorefinery.

8.4 Scenarios for harvesting and transport of grass to processing facility: design and operational-economic analysis

Operations configuration: The grass is cut 3 times during the season. The estimated DM content is set at 18%, and the calculations included 2 levels of yields/ha, high yield of 10 tonnes DM/ha and low yield of 6 DM/ha, respectively (Claus Grøn Sørensen pers. comm., 2021)

All the grass is mowed before harvesting, and the harvesting involves the following harvesting technology:

A. Self-loading wagon with chopping,

B. Self-loading wagon, non-chopping,

C. Self-propelled exact chopper.

Transport configuration: mean transport distance to the plant is set at 10 km for the conventional - and 11 km for the organic clover-grass. The transport from the field to the plant is carried out by lorry or trailer/tractor, with the following load capacities: lorry (55 m^3) and trailer/tractor (40 m^3). The density of the grass was assessed at 365 kg/m^3 for chopped grass and 200 kg/m^3 for non-chopped grass, affecting significantly the load weights of the two systems. The transport speed for the lorry was set at 55 km/h and set at 25 km/h for the trailer/tractor. For the calculations, it is decided only to use lorries for transportation. This is due to general higher transportation costs when transporting the biomass by trailer/tractor (Sopegno et al., 2016; Pavlou et al., 2016).

Calculation scenarios:

Scenario A): mower, self-loading wagon (chopped), unloading device at field exit, lorry transport to plant

Scenario B): mower, self-loading wagon (non-chopped), unloading device at field exit, lorry transport to plant

Scenario C): mower, self-propelled exact chopper, unloading device at field exit, lorry transport to plant

The operational calculations of the harvesting and transportation of grass are based on standard methods for machine performance, costs, etc. (e.g. Sopegno et al., 2016; Pavlou et al., 2016).

In the following the economic assessments of the scenarios are presented:

As described in chapter 4, the green biorefinery is in principle based on cascade utilization of the green biomass. However, in this example the green biorefinery only produces 3 saleable products: soy quality protein, fibre pulp with a feeding value comparable to grass-clover and a low value brown juice which can be used either as a nutrient in crop production or as a raw material in biogas plants.

However, the product mix constitute a number of different price scenarios for the protein concentrate, conventional, non-GM and organic. As for the conventional products, there are no possibilities for a price mark up. For the non-GM products, they are basically identical to the conventional as the raw material (grass-clover) is non–GM (in the EU), but non-GM protein has a bigger price label in some uses where non-GM is in demand. Milk producers delivering to ARLA are demanded to use non-GM feed from now on. This means that non-GM has a higher price in for example Danish and Swedish milk production.

The organic products of protein and fibre pulp have higher prices and the production costs are estimated to be more or less equal.

Production at the biorefinery plant are estimated to be the same for the three product groups, conventional, non-GM and organic (the only difference is raw material cost and transport, which is slightly higher for organic (see tables 8.1, 8.2 and 8.3).

8.5 Economic calculations

The economic assessment encompass the steps from cost of raw material (grass-clover), harvest and transport to the biorefinery plant and calculation of revenue for three scenarios; conventional, non-GM and organic. The raw material cost is based on budget calculations (FarmtalOnline, 2021). Calculation of costs for harvest and logistics is based on standard methods for machine performance. The calculated revenue is based on estimated market prices and production costs. The economic results are stated as the business economic performance for the three-abovementioned scenarios.

8.6 Cost structure

The cost of biomass is estimated at 980.50 DKK/tonne DM for conventional grass-clover and 1,140 DKK for organic grass-clover corresponding to a price of 1.35 DKK/FEN and 1.53 DKK/FEN. This equals the farmer's costs if the grass-clover was used for silage (FarmtalOnline, 2021).

As can be seen from the following tables, the biomass cost (grass-clover) equals around 50% of the cost in all scenarios, and if we add harvest and transport to biorefinery plants, the total cost share for the grass-clover delivered at the biorefinery plant is about 68 – 70 % of the total cost.

The cost of harvest and transport varies between 5,108,521 DKK (self-loading wagon with cutter) - to 6,617,494 DKK (organic grass and self-loading wagon without cutter). The density of the load and the yield are two most important factors affecting the total cost of cost of harvest and transport across the scenarios.

The cost of establishing a biorefinery plant with an annual capacity of 20,000 tonnes DM grass-clover is assumed to be 20,000,000 DKK (Jensen, 2018; Martinsen and Andersen, 2020). This price may very well fluctuate both above and below the set price once the technique is more broadly adopted. The actual cost of establishing the refinery may in the beginning be relatively high, but once the technology is better known and established the construction costs may well decrease. The capital cost is assumed to be 4.5% of the 20,000,000 DKK establishing costs and a 10 year depreciation period.

The selling price of pulp (feeding value) and brown juice (value as biogas) is estimated at the gate by the customer. The transport cost also show that the value of the brown juice cannot pay the transport to a biogas plant, however a localization of the biorefinery together with a biogas plant could be an option.

The price of the protein is based on the market, which can be rather volatile. The price is set at 2,500 DKK/tonne conventional protein, 3,700 DKK/tonne non-GM protein and 5,000 DKK/tonne organic protein.

The price of grass-clover and fibre pulp is based on production price of grass-clover (FarmtalOnline, 2021) and set at 1,350 DKK/FE for the conventional and non-GM grass and 5,000 DKK/FE for the organic fibre pulp.

Lastly, the brown juice is set at 12 DKK/tonnes wet weight (Børgesen et al., 2018).

8.7 Cost and revenue

Tables 8.1, 8.2 and 8.3 show the cost, revenue and economic result for the three described biorefinery scenarios.

Table 8.1. Annual cost, revenue and result in DKK when using a self-loading wagon (chopped)

Cost	Conven-tional	% of cost	Non-GMO	% of cost	Organic	% of cost
Biomass	12,621,797	49%	12,621,797	49%	14,674,740	51%
Harvest	3,652,585	14%	3,652,585	14%	4,313,742	15%
Transport, biomass	1,210,577	5%	1,210,577	5%	1,312,660	5%
Transport, fibre fraction	842,504	3%	842,504	3%	913,549	3%
Transport, brown juice	1,082,819	4%	1,082,819	4%	1,082,819	4%
Processing cost						
Auxiliary cost	727,000	3%	727,000	3%	727,000	3%
Wages	1,474,000	6%	1,474,000	6%	1,474,000	5%
Energy	1,525,000	6%	1,525,000	6%	1,525,000	5%
Maintenance	1,200,000	5%	1,200,000	5%	1,200,000	4%
Capital cost	1,634,000	6%	1,634,000	6%	1,634,000	6%
Total cost	**25,970,282**		**25,970,282**		**28,857,510**	
Revenue						
Protein concentrate	9,445,000		13,978,600		18,890,000	
Fibre fraction	15,074,100		15,074,100		17,083,980	
Brown juice	687,504		687,504		687,504	
Result	**-763,678**		**3,769,922**		**7,803,974**	

Table 8.2. Annual cost, revenue and result in DKK when using a self-loading wagon (non-chopped)

Cost	Conven-tional	% of cost	Non-GMO	% of cost	Organic	% of cost
Biomass	12,621,797	47%	12,621,797	47%	14,674,740	48%
Harvest	2,795,501	10%	2,795,501	10%	4,118,201	13%
Transport, biomass	2,313,021	9%	2,313,021	9%	2,499,293	8%
Transport, fibre fraction	1,609,753	6%	1,609,753	6%	1,739,390	6%
Transport, brown juice	1,082,819	4%	1,082,819	4%	1,082,819	4%
Processing cost						
Auxiliary cost	727,000	3%	727,000	3%	727,000	2%
Wages	1,474,000	5%	1,474,000	5%	1,474,000	5%
Energy	1,525,000	6%	1,525,000	6%	1,525,000	5%
Maintenance	1,200,000	4%	1,200,000	4%	1,200,000	4%
Capital cost	1,634,000	6%	1,634,000	6%	1,634,000	5%
Total cost	**26,982,890**		**26,982,890**		**30,674,443**	
Revenue						
Protein concentrate	9,445,000		13,978,600		18,890,000	
Fibre fraction	15,074,100		15,074,100		17,083,980	
Brown juice	687,504		687,504		687,504	
Result	**-1,776,286**		**2,757,314**		**5,987,041**	

Table 8.3. Annual cost, revenue and result in DKK when using a self-propelled exact chopper

Cost	Conven-tional	% of cost	Non-GMO	% of cost	Organic	% of cost
Biomass	12,621,797	47%	12,621,797	47%	14,674,740	49%
Harvest	4,431,273	17%	4,431,273	17%	5,409,235	18%
Transport, biomass	1,210,577	5%	1,210,577	5%	1,312,660	4%
Transport, fibre fraction	842,504	3%	842,504	3%	913,549	3%
Transport, brown juice	1,082,819	4%	1,082,819	4%	1,082,819	4%
Processing cost						
Auxiliary cost	727,000	3%	727,000	3%	727,000	2%
Wages	1,474,000	6%	1,474,000	6%	1,474,000	5%
Energy	1,525,000	6%	1,525,000	6%	1,525,000	5%
Maintenance	1,200,000	4%	1,200,000	4%	1,200,000	4%
Capital cost	1,634,000	6%	1,634,000	6%	1,634,000	5%
Total cost	**26,748,970**		**26,748,970**		**29,953,003**	
Revenue						
Protein concentrate	9,445,000		13,978,600		18,890,000	
Fibre fraction	15,074,100		15,074,100		17,083,980	
Brown juice	687,504		687,504		687,504	
Result	**-1,542,366**		**2,991,234**		**6,708,481**	

8.8 Economic results

The revenue varies across the 3 scenarios, conventional has the lowest revenue while the nonGM scenario has a higher revenue due to the higher market price for non-GM protein. The organic scenario has the best economic result, the cost of raw material is only slightly higher than for the conventional scenarios and the product price (revenue for both protein and fibre pulp are higher. The price of brown juice is assumed the same for conventional, non-GM and organic.

As table 8.1-8.3 show, a biorefinery based on conventional raw material and selling protein at conventional protein price is not economic viable. The economic result for conventional is negative for all three logistics scenarios, ranging from -1,776,286. DKK to -763,678 DKK.

As can be seen from the table, the non-GM scenario has the same input and processing cost as conventional but the selling price for the protein is assumed to be the higher non-GM price which enables the result to be positive in the range of 2,757,314 DKK to 3,769,922 DKK.

Organic has higher input cost for both biomass and logistics but this is offset by the higher selling prices for organic protein and fibre pulp. The economic result is in the range of 5,987,041 DKK to 7,803,974 DKK.

Based on the above presented results it can be concluded the Non GM scenario and the organic scenario are economic viable due to the higher selling prices.

In Martinsen & Andersen (2020) financial and welfare economic analyses of two scenarios for production of green proteins are presented. The two scenarios feature production of green protein at a biorefinery, integrated with a biogas facility, and with some synergies exploited. Residual biomass resources from the protein production provides input to biogas generation, which in turn supplies process energy for the biorefinery. One scenario features a smaller biorefinery, scaled to an annual grass input of 20,000 tonnes of dry matter, and with only the juice fraction being supplied to the biogas. The other scenario features a large-scale protein plant with an annual grass input of 150,000 tonnes of dry matter. In this case residuals of both juice and fibre are used for biogas generation, with significant investments required for a new biogas plant.

The small-scale biorefinery scenario is similar to the size and production setup illustrated in the present economic assessment apart from the colocation to a biogas plant. The business economic results in Martinsen and Andersen (2020) are similar to the results in the present study where an economic result at approx. –2,035,500 DKK is presented for a conventional small scale biorefinery without any revenue or cost regarding the brown juice, which is fairly similar to the results in this study.

The financial analysis does not include externalities connected to the green biorefinery concept. In order to assess the welfare economic impacts, the study includes a number of relevant externalities.

The externalities considered in the analysis comprise GHG emissions, air pollution, N and P leaching, cadmium as well as road and off-road transport. The small-scale scenario involves positive externalities from reduced N and P leaching as well as from less off-road transport, but the remaining environmental impacts are all negative, with GHG, ammonia and road transport dominating. (Martinsen and Andersen, 2020).

9 Ongoing and concluded commercial, research and development activities

Table 9.1. Overview of support for research and implementation projects on green biorefining in Denmark. The overview has been collected until May 2021 by Danish Protein Innovation (www.proteininnovation.dk) with supplements from the report authors and reviewers.

Projekt	Indhold/content	Links	Deltagere/participants	Tilsagn/funding [m DKK]
Græsprotein				
Demonstrationsprojekt under GUDPs ***Ekstra pulje til fremme af grøn bioraffinering.***				
Kundespecifikt og skræddersyet GræsProtein-anlæg (Tailor-Grass) (2020 – 2023)	AP1 – Etablering af anlæg AP2 – Demonstration af græsproteinfremstilling – fra høst til tørring AP3 – Procesoptimering AP4 – Demonstration og formidling af skræddersyet koncept	https://landbrugsavisen.dk/staten-giver-14-mio-kr-til-stort-græsprotein-anlæg https://mst.dk/service/nyheder/nyhedsarkiv/2020/jan/tailorgrass/	Vestjyllands Andel, Ausumgaard, R&D Engineering & Automation, SEGES	14
Projekter bevilget ved GUDP runde II 2020				
SinProPack	SinProPack ønsker at eftervise anvendelse af græsfibre fra bioraffineret proteinekstraheret grønbiomasse til fremstilling af bæredygtige, formstøbt emballage. Projektet vil udvikle, demonstrere, teste og evaluere fibrebaseret emballage til to-go fødevareprodukter via proof-of-concept, pilotproduktion og industriel opskalering.	Fremtidens kaffe to-go-kop er komposterbar og af græs (mst.dk)	Teknologisk Institut, Aarhus Universitet, LEAF Packaging, COOP, Aarhus Universitet	3.3
HØSTTEK	Høstteknologi med Curru-Tek ApS som producent, Bæredygtige byggeplader med Kronospan ApS og Jena Trading ApS som producenter, Protein fra enggræs med BiomassProtein ApS som producent,	Ny høstteknologi vil forvandle klimaproblem til en bæredygtig forretning (foodbiocluster.dk)	Naturstyrelsen, Food & Bio Cluster Denmark, Curru-Tek ApS, Kronospan ApS, Jena Trading ApS, BiomassProtein ApS, AST A/S, Eiler Chr. Knudsen A/S, Conterra, Københavns	14

Projekt	Indhold/content	Links	Deltagere/participants	Til-sagn/fun ding [m DKK]
	Logistikkæden fra mark til kunde udvikles af AST A/S og Eiler Chr. Knudsen A/S Conterra ApS udvikler et værktøj, som kan inddele lavbundsjorder i forskellige klasser		Universitet, Aalborg Universitet	
Projekter bevilget ved GUDPs program for fremme af grøn bioraffinering				
DLG prototypeanlæg	Etablering af prototypeanlæg Produktion af hvidt protein til humankonsum	https://www.dlg.dk/Om-DLG/Presse/Nyheder/2019/11/DLG-investerer-i-groent-bioraffinaderi-til-klimavenligt-lokalt-produceret-protein	DLG, Biotest, Arla	14
Bæredygtig anvendelse af protein fra grøn biomasse til fødevarer (InnoGrass) (2019-2021)	Oprensningsteknikker Fødevareprodukter Novel-Food-godkendelse	https://www.food.dtu.dk/nyheder/2019/01/graes-skal-goeres-til-en-rentabel-og-sikker-proteinkilde?id=55c942b7-7721-47c2-b539-e5cd65ac569c	DTU, SEGES, Lihme Protein Solutions, AAU, Greenfield Innovation, Naturli', Biotest	4
Lucerne som grovfoder til svin (AlfaMax Bioraf) (2019 – 2021)	1) Forædling for øget mængde råprotein i lucernen. 2) Enzymatisk behandling af planteekstrakt for at frigøre mere protein og forhindre proteinnedbrydning under den efterfølgende proces. 3) Forbedret design af den mekaniske juiceekstraktion fra planten således at plantecellerne åbnes mere effektivt.	https://mst.dk/erhverv/groen-virksomhed/groent-udviklings-og-demonstrationsprogram-gudp/gudp-projekter/2018-projekter/alfamaxbioraf/	DLF, KU, AU	4
Projekter bevilget ved Promilleafgiftsfonden 2020				
Bæredygtig anvendelse af protein fra grøn biomasse til fødevarer (Promilleafgiftsfonden 2020) (Medfinansiering af GUDP InnoGrass)	Se InnoGrass ovenfor		SEGES	0.2

Projekt	Indhold/content	Links	Deltagere/participants	Tilsagn/funding [m DKK]
Opskalering og validering af processer for separering af restsaft fra produktion af græsprotein (Promilleafgiftsfonden 2020)	Projektet har til formål at udvikle det tekniske potentiale i opkoncentrering af restsaft fra produktion af græs protein i nær fuldskala, samt skabe et solidt grundlag for vurdering af det økonomiske potentiale. Målet er at optimere og validere membranfiltreringen i demonstrationsskala, samt skabe tilstrækkelig værdi af koncentrat og permeat, så produktion af græs protein bliver økonomisk rentabelt. Opkoncentrering/membranfiltrering af sukkersfoffer i brunsaft.		AU	1.2
Igangværende projekter				
GrassTools – Tools for improving grassland biomass production and delivering multiple ecosystem services (IFD 2021-2026)	GrassTools aims to develop mechanistic insight and tools to optimize farmer's grassland management and secure documentation for future regulation. The project will investigate the factors determining climatic and environmental effects of a transition from cereal-based crop rotations to perennial grassland systems in order to enhance the societal value of the change.	www.grasstools.dk	Arla, Danish Crown, Yara, DSV frø, Lemvigegnens farmers Union, Skive Municipality, Ministry of Food, Agriculture and Fisheries, Vestjyllands Andel and Aarhus University	19.4
Græs4Food: Udvikling af en membranproces til raffinering af højkvalitets fødevareprotein fra kløvergræs og lucerne (2020 – 2023)	Udvikling af en proces til højkvalitets fødevareprotein fra kløvergræs og lucerne, som skal optimeres og opskaleres i pilotskala.	https://mst.dk/erhverv/groen-virksomhed/groent-udviklings-og-demonstrationsprogram-gudp/gudp-projekter/2020-projekter/graes4food/	BiomassProtein, Aalborg Universitet, MMS Nordic, GreenLab Skive, Thise Mejeri	8.1

Projekt	Indhold/content	Links	Deltagere/participants	Tilsagn/funding [m DKK]
	Det samlede koncept, inkl. håndtering af sidestrømme, skal integreres i en BiomassProtein™ protein fabrik i GreenLab Skive. Udviklingen af fødevareproteinprocessen vil ske i et tæt samarbejde mellem BiomassProtein, Center for Membranfiltrering ved Aalborg Universitet, membranfiltrerings-firmaet MMS, GreenLab Skive og Thise Mejeri. De producerede fødevareproteiner vil blive analyseret for fødevare-egenskaber og benyttet til udvikling af nye fødevareprodukter.			
Værdiskabelse med græsprotein (Græs-prof) (2020 – 2023)	AP1 – sorter og markforsøg: AP2 - Høstteknik: AP3 - Neddelingsteknik: AP4 - Højværdi – Tanniner fodertilskud AP5 – Bæredygtighed / LCA / PEF	https://www.seges.dk/innovation-og-udvikling/graesprotein	DLF Seeds, SEGES, Kverneland, Maxi-Grass, AU, AAU SDU, Vestjyllands Andel Vinderup Maskinforretning, Maskinstation Martin Børsting	11.3
Grass Biochar - Energi fra pressepulp fra græsprotein-produktion til drift af raffineringsprocessen og produktion af værdifuldt biokul (2020 - 2022)	AP1: Test af græspulp på DTP processen AP2: Design, installation og integration AP3: Kvalitet og anvendelse af græs-biochar AP4: Procesanalyse, klima og energiregnskab AP6: Projektledelse, administration og kommunikation	https://mst.dk/erhverv/groen-virksomhed/groent-udviklings-og-demonstrationsprogram-gudp/gudp-projekter/2019-projekter/2-grass-biochar/	AU, Norphos, Aquagreen, DTU, RUC	6.0

Projekt	Indhold/content	Links	Deltagere/participants	Tilsagn/funding [m DKK]
GræsProteinFoder: Enzym optimeret græsprotein til unge produktionsdyr (GUDP) (2019 – 2023)	Formålet med projektet er at udvikle et forbedret græsproteinprodukt fra dansk produceret plantemateriale til helt unge produktionsdyr. Projektet vil fokusere på at forbedre proteinproduktet gennem designede enzymatiske behandlinger, der vil resultere i foder, der har forøget fordøjelighed for dyrene og som indeholder færre antinæringsstoffer	https://mst.dk/erhverv/groen-virksomhed/groent-udviklings-og-demonstrationsprogram-gudp/gudp-projekter/2019-projekter/graesproteinfoder/	AAU, KU, BiomassProtein, Glucospot, Hamlet Protein, DLF	8
GO-GRASS (H2020) (2019 – 2023)	Udvikling af demoanlæg fokus på samarbejde med Velas. Udvikling af paludikultur som en mulig leverandør af råvarer til bioraffinering	https://www.go-grass.eu/	AU, Agro Business Park, Velas, INVESTORNET-GATE2GROWTH, + en lang række udenlandske partnere	74 (11 for AU)
GreenVALLeys (2019 – 2022 (Interreg)	1) Systemanalyser 2) Bæredygtighedsanalyser 3) Etablering af pilotanlæg i Sverige	https://agrovast.se/eu-projekt/green-valleys/ https://cbio.au.dk/nyhed/vis/artikel/nyt-dansk-svensk-projekt-med-fokus-paa-graes-og-groen-bioraffinering/	AU, Chalmers, Skive Kommune, Agroväst, Sveriges Lantbruksuniversitet, Hushållningssällskapet Sjuhräd, Vestra Götlandsregionen	37.5
PALUDI-fiber (Gluds Legat)	The objectives are to - obtain more knowledge for optimal establishment and management of alternative flooding tolerant crops - characterize the fibre fraction from the side stream when leaf protein is extracted in biorefineries	https://pure.au.dk/portal/en/projects/baeredygtige-fibre-fra-biomasse-produceret-paa-kulstofrige-og-vaade-lavbundsjorde(b1a85bde-5017-41ba-ad16-179b7dc90e83).html	AU	0.7

Projekt	Indhold/content	Links	Deltagere/participants	Tilsagn/funding [m DKK]
	- investigate how the fibre composition depends on the nutrient status, harvest time and frequency of the crop			
Dansk demoskala teknologiplatform for forskning i grøn biomasse (GRØNBIORAF) (GUDP + Region Midt + Arla, Danish Crown, DLG, DLF) (2018 – 2021)	1) Etablering og test af demoanlæg på Foulum 2) Kortlægning af effekter 3) Kommercialisering og forretningsudvikling	https://mst.dk/erhverv/groen-virksomhed/groent-udviklings-og-demonstrationsprogram-gudp/gudp-projekter/2017-projekter/groenbioraf/	Aarhus Universitet Engineering, Københavns Universitet, Agro Business Park	8 (GUDP) 3.5 (Region Midt)
Afsluttede projekter/salgsopgaver				
Konsulentopgaver	1) Rentabilitetsanalyse ved grøn bioraffinering for Rybjerg Biogas 2) Bornholm - griseproduktion		SEGES	
Optimal udnyttelse af bioraffineret pulp fra grøn biomasse til kvægfoder (Hofmansgavefonden) (2019 – 2020)	1) Fodringsforsøg med presseret fra grøn bioraffineriring 2) Økonomiberegninger		KU, AU, SEGES	1.5
CSR-PORK 4.0 (GUDP) (2016 – 2020)	1) Fodringsforsøg med græsprotein til konventionelle grise 2) Bæredygtighedsvurderinger	https://mst.dk/erhverv/groen-virksomhed/groent-udviklings-og-demonstrationsprogram-gudp/gudp-projekter/2016-projekter/dokumenteret-baeredygtighed-og-ressourceoptimering-i-hele-svinekoedets-vaerdikaede-csr-pork-40/	SEGES, Aarhus Universitet, Danish Crown	7.1
Subleem 2.0 (GUDP) (2019 – 2020)	I projektet etableres et fødevaregodkendt pilotskala biomasse separationsanlæg til forarbejdning af grøn biomasse.	https://www.teknologisk.dk/projekter/projekt-subleem-2-0/39276	Teknologisk Institut, Nordic sugar, Københavns Universitet, KU-	2.8

Projekt	Indhold/content	Links	Deltagere/participants	Tilsagn/funding [m DKK]
			FOOD, KWS Scandinavia A/S, Green Solutions	
Bioraf-Business (Svine- og Fjerkræafgiftsfonden) (2018-19)	1) Analyse af afgrødefordeling / placeringsmuligheder 2) Økonomikalkuler 3) Case-analyser	https://www.seges.dk/da-dk/nyheder/seges%20flytter%20gront%20protein%20fra%20tegnebrattet%20til%20virkeligheden	SEGES	0.4
Græsprotein-fabrik (Innovationssamarbejde under Region Midtjyllands Udviklingsprogram for Bioøkonomi) (2018-19)	Udvikling af investeringsoplæg til bygning og idrifttagning af BiomassProtein proteinfabrik i GreenLab Skive.	https://www.rmbio.dk/nyheder/ny-rapport-om-graesprotein?Action=1&M=NewsV2&PID=13439	R&D Engineering, SiccaDania, AU, Danish Marine Protein, RUNI, Skive Biogas, Vestjyllands Andel, Nordic Seaweed	0.9
BiomassProtein (Innovationssamarbejde under Region Midtjyllands Udviklingsprogram for Bioøkonomi) (2018-19)	Projektering af græsproteinfabrikker (mobile anlæg) Case med DMP i Skive	https://www.rmbio.dk/cases/biomass-protein-green-lab-skive	AAU, BiomassProtein, GreenLab Skive, Greenetic Aps, Aktive Energianlæg	1
SuperGrassPork (GUDP / Organic RDD3) (2017-19)	1) Teknisk optimering (Høsttid, dobbeltpresning, lagringsforsøg) 2) Fodringsforsøg med slagtegrise 3) Bæredygtighed (miljø og økonomi) 4) Interessentanalyse 5) Økonomiske effekter / Roadmap	https://icrofs.dk/forskning/dansk-forskning/organic-rdd-3/supergrass-pork/	SEGES, Aarhus Universitet, Aalborg Universitet, IFAU, KU, Friland A/S	6.7

Projekt	Indhold/content	Links	Deltagere/participants	Tilsagn/funding [m DKK]
GreenEggs (GUDP) (2017-20)	Udvidede forsøg med græsprotein til æglæggere – æg-kvalitet Forsøg med at bruge pileblade fra træer i hønsegård.	https://mst.dk/erhverv/groen-virksomhed/groent-udviklings-og-demonstrationsprogram-gudp/gudp-projekter/2016-projekter/green-egss/	Aarhus Universitet Aalborg Universitet, Ingeborg og Brian Holm, DLG, SEGES	7.8
BioValue (Innovationsfonden) (2014-18)	Tre arbejdspakker: 1) Biomasser 2) Proteinudvinding 3) SESE Platform		Aarhus Universitet, Aalborg Universitet, Københavns Universitet SEGES, KMC, Arla, DLF (WP1+ WP2 om dyrkning af græs og grøn bioraffinering + en række partnere til øvrige arbejdspakker)	79.5
OrganoFinery (GUDP / Organic RDD2) (2014-18)	1) Plantearter / proteinudbytte 2) Bioraffinering med mælkesyrefermentering 3) Fodringsforsøg med æglæggere 4) Biogaspotentiale 5) Praktisk anvendelse 6) Markedsvurdering 7) Økonomi	https://icrofs.dk/forskning/dansk-forskning/organic-rdd-2/organofinery/	Aalborg Universitet, SEGES, Fermentationsexperts A/S, Københavns Universitet, Aarhus Universitet, Biotest ApS, IFAU og Teknologisk Institut-AgroTech	10
MultiPlant (GUDP / Organic RDD2) (2014 – 2018)	1) Planteartsblandinger – udbytte 2) Effekt på insekter 3) Forsøg med kyllinger 4) Biogaspotentiale 5) Økonomi / bæredygtighed	https://icrofs.dk/forskning/dansk-forskning/organic-rdd-2/multiplant/	Aarhus Universitet, Økologisk Landsforening, SEGES, Agro Business Park A/S, DLF-Trifolium PlanEnergi Københavns Universitet Vestjyllands Andel	9
BioBase (2014 – 2017)	1) Dyrkningssystemer på sædskiftearealer 2) Biomasse fra engarealer	https://dca.au.dk/forskning/biooekonomi-og-biobaseret-produktion/biobase/	Aarhus Universitet	45

Projekt	Indhold/content	Links	Deltagere/participants	Tilsagn/funding [m DKK]
	3) Grøn Protein 4) Produktion af bio-olie med HTL-teknologi 5) Konsekvenser af øget biomasseproduktion			
Opskalering og validering af processer for separering af restsaft fra produktion af græsprotein (Promilleafgiftsfonden) (2019)	Projektet har til formål at udvikle nær fuldskala processer for at koncentrere restsaft fra produktion af græsprotein ('grønt protein') samt at evaluere udnyttelsespotentialet af både koncentrat og permeat.	https://promilleafgiftsfonden.dk/det-har-fonden-stoettet/bevillingsoversigter/fondens-bevillinger-2019/aarhus-universitet	Aarhus Universitet	1.9
Produktion, anvendelse og økonomisætning af koncentreret restsaft fra produktion af græsprotein (Promilleafgiftsfonden) (2018)	Projektet har til formål at validere muligheden for at koncentrere restsaft fra produktion af græsprotein ('grønt protein') samt vurdere det økonomiske potentiale i en sådan produktstrøm	https://promilleafgiftsfonden.dk/det-har-fonden-stoettet/bevillingsoversigter/fondens-bevillinger-2018/aarhus-universitet	Aarhus Universitet	1.5

Projekt	Indhold/content	Links	Deltagere/participants	Tilsagn/funding [m DKK]
BIOREF (Det strategiske forksningsråd) (2009 – 2013)	1) proteinudvinding fra grønsaft af lucerne ved hjælp af skruepresning og efterfølgende udfældning med svovlsyre 2) anvendelse af presserest til produktion af værdistoffer gennem forbehandling og hydrolyse 3) karakterisering af lignin-fraktionen med henblik på identificering af antimikrobielle egenskaber (anti-biofilm) 4) at identificere, udvikle og anvende on-site svampeenzymer til enzymatisk hydrolyse 5) at anvende produceret sukker til produktion af bioethanol (via gærfermentering) 6) til produktion af biokemikalier i form af organiske syrer (svampefermentering)		AAU, KU, Biogasol Aps, Solum A/S, Biotest Aps-	16.7
BiomassProtein™; udvikling af mobilt anlæg til udvindelse af funktionelle proteiner fra grønne planter (græs, kløver mm.) (Innobooster) (2018)	1) Udvikling af mobilenhed		BiomassProtein	0.4

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About DCA

DCA - Danish Centre for Food and Agriculture is the entrance to research in food and agriculture at Aarhus University (AU).

The Centre comprises AU departments with food and agricultural science activities. These are primarily Department of Agroecology, Department of Animal Science, Department of Food Science, Centre for Quantitative Genetics and Genomics, and parts of Department of Engineering.

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SUMMARY

Green biomass may be used to produce local protein, and substitute other protein sources, and at the same time obtain environmental benefits. Grass or grass-clover crops on arable land can deliver high yields of biomass as well as protein with a good amino acid profile. Grass from unfertilized permanent grassland may represent an opportunity if focus is on the fibre part of the grass. For cover crops to be an attractive supply of biomass, production systems need to be developed with a sufficiently high production to cover harvesting costs. Changing from wheat or maize to grass results in decreased N-leaching and greenhouse gas emissions. With current techniques, 40% of the protein in the green biomass can be recovered in a protein concentrate with protein content around 50% of dry matter, similar to soybean meal. Higher contents are possible for specialty applications. In addition, a fibre fraction containing 15-18% protein can be produced and used for ruminant feed, bioenergy production or further biorefined into chemical building blocks or used for bio-materials. Experiments have been performed on several animal species, where soy was replaced without negative effects on animal performance. High contents of unsaturated fat in the protein affect the meat and fat tissue and may be a limiting factor for the amount of included green protein. The fibre fraction seems suitable for ruminant feeding replacing other types of silages. The first industrial scale biorefineries on green biomass for feed and bioenergy are now established in Denmark, while more research is needed to evaluate the protein quality for food applications, and in addition a full EFSA approval. There are major uncertainties in the economic assessment of establishing a full-scale biorefinery. Major obstacles are transportation costs and uncertainty in running cost for the biorefinery. The largest prospects are within the organic sector where there is a need for locally sourced, sustainable protein.