FEED-A-GENE

Adapting the feed, the animal and the feeding techniques to improve the efficiency and sustainability of monogastric livestock production systems

Deliverable D1.2

New process for the extraction of protein from green biomass

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Classified, as referred to in Commission Decision 2001/844/EC - CI	

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1. Summary

Objectives

The objective of task 1.2 was to optimize the extraction conditions for protein from green biomass in a pilot plant. The experiments involved extraction of protein from ryegrass, clover, and legume without and with the addition of cell wall degrading enzymes during processing. We also studied technologies for the elimination of anti-nutritional compounds. The deliverable is based on activities in WP1 task 1.2 and has strong links to tasks 1.4, 1.5, and 1.6.

Rationale:

Task 1.2 focused on optimizing the extractions conditions for protein from green biomass (i.e., ryegrass, clover, legumes) in a pilot plant. The green biomass was separated into two main streams – a liquid stream composed of soluble proteins, carbohydrates and minerals, and a fibre-rich solid stream that contains the majority of cell walls and insoluble proteins. The protein in the liquid stream was precipitated by acid following spontaneous fermentation or by heat (i.e., heat exchange, steam precipitation), and finally freeze-drying. Cell wall degrading enzymes (i.e, cellulases, xylanases, and pectinases) were selected in vitro and used to study the potential to increase the yield of protein in the pilot plant. Furthermore, the removal of antinutritional compounds in protein concentrates precipitated by acid and steam was studied. The protein concentrates and the pulp were analysed for ash, fat, protein, amino acids, and carbohydrates and the digestibility of protein in the concentrates was evaluated in vitro using the pH-stat method. The protein concentrates produced in task 1.2 were evaluated nutritionally in pigs in task 1.4 and the pulp in task 1.5.

Teams involved:

AU
DuPont
Hamlet Protein
DLO

Species and production systems considered:

All animal species and countries in Europe, feed industry.

2. Introduction

On a global scale, population and income growth in developing countries are fuelling a greater per capita consumption of animal protein. Currently, soybean meal derived primarily from North and South America has been the primary protein source for animal feed. However, public, environmental, and health concerns in the production countries and the desire for European protein autonomy have increased the search for sustainable alternative protein sources. In temperate climates, green crops such as grass and legumes have the potential to become such sources. These forages produce high yields of dry matter (DM) and crude protein (CP), and have an amino acid composition similar to that of soya (Damborg, 2019).

In green biomass, proteins are mainly concentrated in plant leaves and previous research has primarily focused on extracting proteins from the green leaves. Past studies have worked on processes that fractionate green leaves into a liquid fraction (juice) containing soluble proteins and a fibrous solid fraction (pulp) (Colas et al., 2013). The juice can be further processed into a protein-rich concentrate, thus removing a large proportion of unwanted components. It has been possible with this technology to extract a green protein filtrate from lucerne containing 51% of the original plant protein (Colas et al., 2013).

National strategic and innovation activities (i.e., the Biovalue project; https://biovalue.dk) and local activities at Aarhus University (i.e., BIOBASE; http://dca.au.dk/en/research/bioeconomy-and-biobased-production/biobase/) have focused on developing the bioeconomy. The activities in task 1.2 of the Feed-a-Gene project have been complementary to the BIOBASE activities by focusing on optimizing the conditions for extraction of protein from green biomass (i.e., ryegrass, clover, legumes) in a pilot plant. The pilot plant was developed during the course of the Feed-a-Gene project and its configuration and operational conditions were based on results obtained from laboratory extraction experiment funded by the BIOBASE project of Aarhus University. Published results from lab-scale extraction and other activities can be found elsewhere (Stødkilde et al., 2018; Damborg, 2019; Stødkilde et al., 2019).

3. Results

As mentioned above, the activities in task 1.2 have been coordinated with national Danish projects, which started back in 2012 and will continue beyond the activities of Feed-a-Gene (Figure 1). The focus in Feed-a-Gene has been on optimizing the conditions at pilot-scale extractions performed during the years 2016-2018. The protein extracted in 2016 and 2017 was studied in task 1.4 and the pulp after the extraction of the protein was used in an experiment with rabbits in task 1.5. Extracts obtained from the harvest in 2018 was analysed chemically but not nutritionally evaluated during the Feed-a-Gene project.

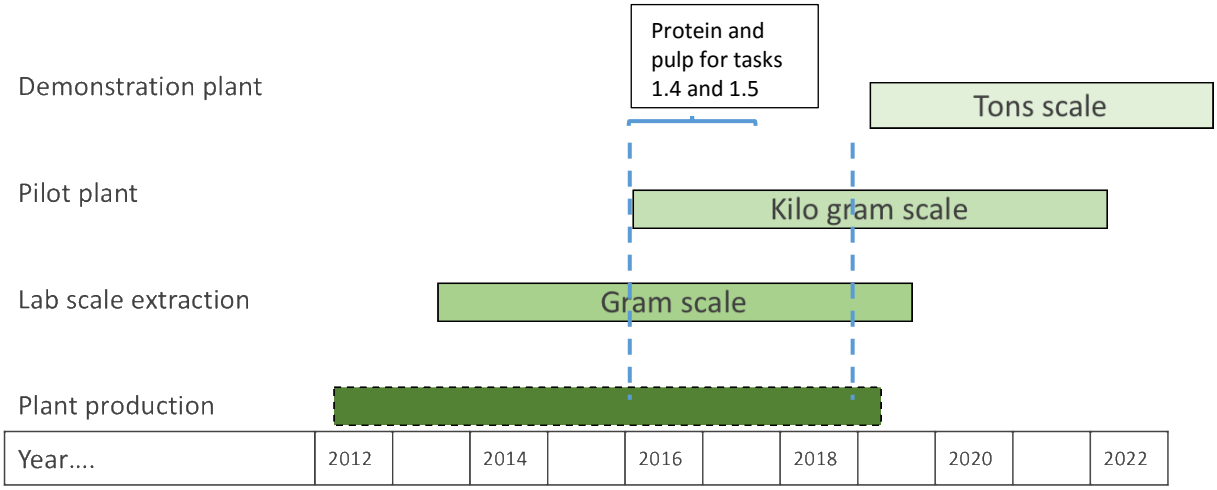


Figure 1. Overview of national and Feed-a-Gene activities on extraction of protein from green biomass.

3.1 Pilot plant for processing of green biomass

The flow-sheet of the pilot plant at Aarhus University is illustrated in Fugure 2. The pilot plant consist of an input devise, a shredder for maceration, a single or double screw press for separation of the juice from the pulp, an accumulator tank that also is used when the protein is acid-precipitated by spontaneous fermentation, a heat exchanger when protein is precipitated by heat, a decanter centrifuge for separating the protein concentrate from the residual juice, and finally a drying step for removing the moisture from the protein paste. Freeze-drying of material from the industrial plant has been used to remove the moisture in the protein paste for the samples used in the Feed-a-Gene project. After the fractionation and before the juice reached the tank, it passed through a sand trap for removal of sand and other heavy impurities.

The configuration of the pilot plant has been changed gradually over time. In 2016, the fractionation of the juice from the pulp was performed on a single screw press whereas from 2017 onwards a double screw press was used. In 2016, the protein was precipitated by heat using a heat exchanger, but it was changed to acid precipitation through spontaneous fermentation due to technical problems in obtaining efficient precipitation. Fermentation was also used in 2017. In 2018, heat precipitation by steam was introduced to replace fermentation.

The procedure for harvesting the green biomass underwent changes during the years. In 2016 and 2017, the green biomass was cut and air-dried on the field before being collected, whereas in 2018 the green biomass was cut and collected directly.

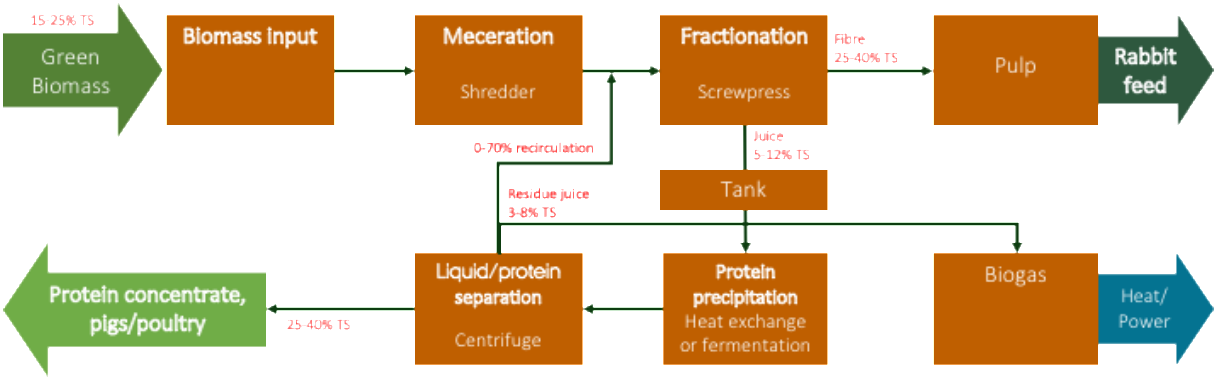


Figure 2. The principles of the pilot plant for the processing of green biomass into protein and pulp.

3.2 Selection of enzymes for green biomass

The main objective for enzymatic treatment of the green biomass during processing was to obtain a higher protein concentration and improve the digestibility of the protein. One of the reasons for a low concentration of protein in the concentrate was due to high concentrations of fibres. To guide the selection of fibre-degrading enzymes for the process, the following criteria were considered:

1. Composition of the recalcitrant fibres in the green biomass
2. Enzymes needed to be available in sufficient amounts
3. Enzymes should be applicable for animal nutrition.

Based on these criteria, a selection of enzymes was made. The enzymes had degradation activities for substrates like pectins, hemicellulose (including beta-glucans), cellulose, xylo-glucans, and arabinogalactans.

To select the most efficient enzyme combination, a model system reflecting the pilot scale processing facility was set up. Using the same biomass substrate as in the pilot scale, different combinations and dosages of enzymes were tested. The combination of enzymes tested were designed so that each combination contained enzymes active on pectins, hemicellulose, cellulose, xylo-glucan, and arabinogalactans, but of different microbial origin. This was typically done by combining three enzyme products. The selected enzyme and combinations were dosed at commercial relevant dosages. To evaluate the performance of the selected enzymes, a range of assays to determine fibre degradation and protein solubilisation was implemented (Pedersen et al., 2015). Based on this initial screening, changes in the protein/carbohydrate ratio could be determined. The enzyme combinations tested varied in efficiency and the best combination found gave an increase of 15 to 30% in the protein/carbohydrate ratio, depending on the biomass used (i.e., for red clover and ryegrass). The increased purity of the protein fraction was driven by a decrease in total sugars after the enzyme hydrolysis. The best combination of enzymes was used for pilot scale application.

3.3 Composition of protein and pulp extracted from green biomass without and with enzymes

3.3.1 Products obtained in 2016

Year 2016 was the first year where the pilot plant at Aarhus University was in operation. The fractionation was performed on a single screw press and the protein separated from the juice by either heat precipitation on a heat exchanger or by fermentation. Because Aarhus University was committed to process green biomass for other projects (e.g., for the Biovalue project) and technical problems during the start-up phase, the green biomass for the Feed-a-Gene project could not be processed until August-September 2016. While sufficient protein was produced in one batch for ryegrass without and with enzymes and for red clover without enzymes, two batches were needed for obtaining a sufficient amount of protein from red clover with enzymes. The two batches were produced after a dry and a rainy day, respectively. The two batches were mixed in a ratio 26:74, and the result of the mixed batches are reported in Tables 1 through 3 along with the data for the other batches. Detailed data are given in Annex 1.

The samples were analysed for the chemical composition and in vitro digestibility of the protein. The dry matter (DM) content was determined by drying the samples at 103˚C to a constant weight and ash was analysed according to the AOAC method (923.03; AOAC) (Association of Official Analytical Chemists, 1990). Nitrogen was measured by the Dumas method and was protein calculated as N×6.25 (Hansen, 1989). Fat was determined using the Stoldt procedure (Stoldt, 1952) and sugars (i.e., glucose, fructose and sucrose) and fructans were determined as described by Larsson and Bengtsson (1983). The dietary fibre content and composition was analysed by an enzymatic-chemical-gravimetric method (Bach Knudsen, 1997). This method is based on the removal of sugars and starch and the determination of soluble non-cellulosic polysaccharides (NCP), insoluble NCP, cellulose, and total non-starch polysaccharides (NSP) based on the monomeric constituents of the fibre matrix and gravimetric determination of Klason lignin (acid insoluble residue) (Bach Knudsen, 1997), but modified to include measurements of low-molecular weight, non-digestible carbohydrates (LMW-NDC) (Lærke et al., 2015). The kinetics of protein degradation were determined by the degree of hydrolysis using a modification of the method described by Pedersen and Eggum (1983) using the pH-stat method (Butré et al., 2012).

The protein content of the processed ryegrass and red clover was 15.3 and 21.5% of DM, respectively, whereas the protein content of the concentrates extracted without and with the use of cell wall degrading enzymes was approximately the same, around 33% of DM (Table 1). However, if the two batches of red clover with added enzymes had been treated separately, the protein content would have been more variable (28.2 and 35.7% of DM) primarily caused by a big difference in the ash content (11.1 and 20.7% of DM). The amino acid composition was also similar in all four fractions produced (Table 2) and similar to what has been found in laboratory scale extraction studies (Damborg, 2019). The ash content was high and variable

(13.6 to 30.5% of DM). The content of Klason lignin (15.0 to 17.7% of DM) and fat (4.3 to 7.6% of DM) was also high but the variation was much less. The content of total carbohydrates (Total-CHO) was 16.0 and 18.0% of DM of ryegrass and red clover concentrates extracted without the use of cell wall degrading enzymes. For the two concentrates extracted with the use of cell wall degrading enzymes, Total-CHO was only approximately half in the protein concentrate from ryegrass. The effect of the cell wall degrading enzymes on total carbohydrates in protein concentrate from red clover was much less. Details concerning the carbohydrate composition are given in Annex 2.

Table 1. Main chemical composition of protein from ryegrass and red clover extracted in 2016 without and with the use of enzymes.

Green biomass	**Ryegrass**		**Red clover**	
Enzymes	No	Yes	No	Yes
Precipitation	Heat	Heat	Fermentation	Fermentation
Dry matter, %	98.6	98.2	97.9	96.3
	Values, % of dry matter			
Ash	23.0	30.5	20.1	13,6
Protein (Nx6.25)	33.0	33.4	33.2	33.7
Fat	6.6	4.3	7.6	7.2
Total-CHO	16.0	8,7	18.0	16.1
Sugars	0.6	0.1	1.4	0.8
Starch	1.3	0.8	1.0	1.0
Fructans	1.6	0.0	2.6	1.1
LMW-NDC	2.8	1.0	2.5	2.8
S-NCP	1.7	2.6	1.7	2.1
I-NCP	4.7	1.9	4.7	4.5
Cellulose	3.2	2.3	4.1	3.9
Klason lignin	17.3	17.7	16.0	15.0

Total-CHO, total carbohydrates; LMW-NDC, low-molecular weight non-digestible carbohydrates; S-NCP, soluble non-cellulosic polysaccharides; I-NCP, insoluble non-cellulosic polysaccharides.

Table 2. Amino acid composition of protein from ryegrass and red clover extracted in 2016 without and with the use of enzymes.

Green biomass	**Ryegrass**		**Red clover**	
Enzymes	No	Yes	No	Yes
Precipitation	Heat	Heat	Fermentation	Fermentation
	Indispensable amino acids, g/100 g crude protein			
Lys	5.83	5.85	5.74	5.80
Met	1.81	1.65	1.82	1.80
Cys	0.72	0.77	0.70	0.68
Thr	4.42	4.34	4.41	4.15
Trp	1.98	1.91	2.02	1.89
Ile	4.97	4.91	4.96	4.89
Leu	8.18	8.00	8.20	7.74
His	2.28	2.32	2.27	2.17
Phe	5.50	5.39	5.61	5.22
Val	6.17	6.10	6.14	5.98
Arg	5.49	5.36	5.38	5.10
	Dispensable amino acids, g/100 g crude protein			
Ala	5.68	5.53	5.69	5.27
Asp	9.11	8.84	8.94	8.73
Glu	9.96	9.63	9.90	9.70
Gly	5.11	5.03	5.11	5.08
Ser	4.24	4.11	4.19	3.96
Tyr	4.16	4.22	4.13	3.84

Of the pulps produced in 2016, only ryegrass extracted without and with the use of cell wall degrading enzymes was used in the rabbit experiment in task 1.5. The ash content was higher in pulp from red clover than in pulp from ryegrass whereas the opposite was true for Total-CHO, which was around 10 percentage units higher in ryegrass than in red clover (Table 3 and Annex 3). The higher content of carbohydrates in pulp from ryegrass concerned particularly fructans, cellulose, and I-NCP, whereas the opposite was the case for S-NCP and LMW-NDC.

Table 3. Main chemical composition of pulp after extraction of protein from ryegrass and red clover extracted in 2016 without and with the use of enzymes.

Green biomass	Ryegrass		Red clover	
Enzymes	No	Yes	No	Yes
Drying	Air	Air	Freeze-dried	Freeze-dried
Dry matter, %	94.5	93.9	97.9	97.6
	Values, % of dry matter			
Ash	8.5	7.5	12.8	12.9
Protein (Nx6.25)	10.1	9.7	nm	nm
Fat	1.7	2.6	nm	nm
Total-CHO	64.4	65.9	51.1	53.5
Sugars	1.0	1.3	2.5	1.8
Starch	0.7	0.9	0.2	0.0
Fructans	2.9	3.4	0.0	0.0
LMW-NDC	4.5	5.1	6.4	7.1
S-NCP	4.2	2.5	8.0	8.0
I-NCP	21.8	23.6	13.2	13.9
Cellulose	29.4	29.1	20.8	22.7
Klason lignin	11.9	11.3	12.6	15.2

nm, not measured; Total-CHO, total carbohydrates; LMW-NDC, low-molecular weight non-digestible carbohydrates; S-NCP, soluble non-cellulosic polysaccharides; I-NCP, insoluble non-cellulosic polysaccharides.

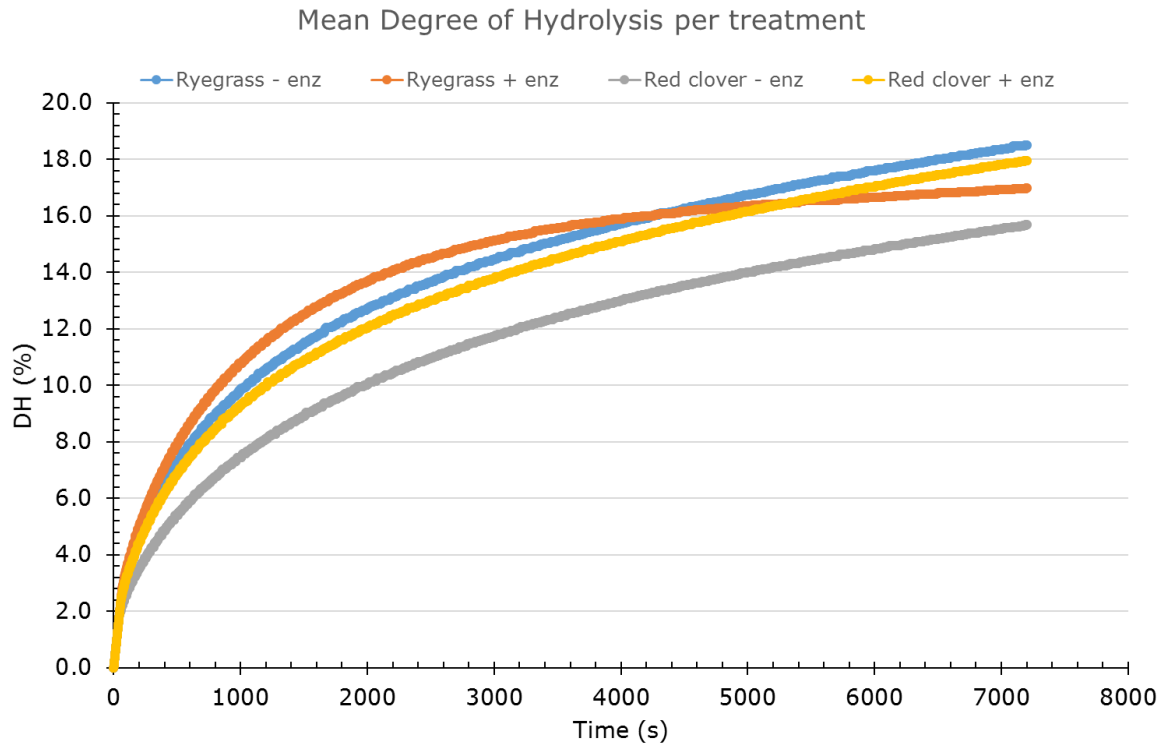


Figure 3. Degree of hydrolysis determined with the pH-stat method of protein from ryegrass and red clover extracted without and with the use cell wall degrading enzymes.

In Figure 3, the degree of protein hydrolysis determined with the pH-stat method is shown for protein from ryegrass and red clover extracted without and with the use of cell wall degrading

enzymes. Without the use cell wall degrading enzymes, the rate constant (i.e., determining the shape of the curve) was similar for ryegrass and clover, whereas the maximum degree of hydrolysis was higher for ryegrass than for red clover. Enzyme treatment during protein extraction enhanced the degree of hydrolysis of both ryegrass and red clover, but in a differential way. In ryegrass, enzyme treatment increased the rate of hydrolysis, as indicated by the steeper initial slope of the curve, whereas in red clover the enzyme treatment increased the maximum degree of hydrolysis (see Annex 5 for details). The rapid initial increase in the degree of hydrolysis of enzyme-treated ryegrass may be related to the reduction in total and fibrous carbohydrates as indicated in Table 1. The results of the in vivo studies with ileal-cannulated pigs are reported in Deliverables 1.4 and 1.5.

3.3.2 Products obtained in 2017

Based on the experience from the first year of operation, a number of improvements were implemented on the pilot plant for 2017 including a more efficient input system, better maceration of the biomass, installation of double pressing, better handling of the juice for reduced foam formation, and optimization of the pumping system. Particularly the installation of double pressing was expected to improve the protein yield as a laboratory scale experiment with double pressing of lucerne had shown that the yield of protein in the liquid fraction could be improved from 40 to 70% of the original protein in the green biomass (unpublished results).

The three green biomasses that were processed in 2017 were ryegrass, red clover, and lucerne. The protein from ryegrass was produced on three production days from the end of May until the beginning of June and again one day in August. Lucerne was produced on three production days in June, one production day in July and two production days in August. Red clover was produced on one production day in August. It was planned that all sub-batches be treated separately in the industrial freeze-drying plant but due to a mistake of the drying company, the sub-batches were mixed within plant origin, making it impossible to study the variability from batch to batch.

Table 4. Main chemical composition of protein from ryegrass, red clover and lucerne extracted in 2017 compared with soybean meal.

Green biomass	Ryegrass	Red clover	Lucerne	Soybean meal
Precipitation	Fermentation	Fermentation	Fermentation	
Dry matter, %	98.4	99.4	98.8	90.3
	Values, % of dry matter			
Ash	16.9	29.4	14.8	7.7
Protein (Nx6.25)	35.7	33.0	37.8	55.2
Fat				
Total-CHO	17.5	9.9	18.9	31.9
Sugars	0.6	0.1	0.1	5.2
Starch	0.7	2.2	4.1	0.4
Fructans	0.5	0.1	0.1	7.8
LMW-NDC	2.4	1.9	3.9	1.7
S-NCP	1.8	1.5	3.1	3.9
I-NCP	6.3	2.8	3.8	10.0
Cellulose	5.2	1.4	3.9	3.1
Klason lignin	19.3	21.1	20.9	4.3

Total-CHO, total carbohydrates; LMW-NDC, low-molecular weight non-digestible carbohydrates; S-NCP, soluble non-cellulosic polysaccharides; I-NCP, insoluble non-cellulosic polysaccharides.

Although double screw press was installed in the pilot plant in 2017 and results from lab-scale experiments showed higher yield and concentration of protein in the concentrate, the results of the pilot plant did not live up to our expectations (Table 4 and Annex 5). Compared to the protein content of the protein concentrates processed in 2016 (Table 1), the increase in protein content (i.e., from 33.0 to 35.7% of DM) in the concentrates from ryegrass and red clover was modest and it was only in the protein concentrate from lucerne that there was a tendency for a higher content (37.8% of DM). All the protein concentrates processed in 2017 had a high ash content, as was the case in 2016, and much higher than in soybean meal. It is clear from Table 4 that the protein content in all concentrates is substantially lower than in soybean meal, mostly caused by the high ash content and inefficient precipitation of protein by fermentation. The amino acid composition, however, is similar to what is reported for soybean meal. Lysine and sulphur containing amino acids are in the same range and threonine and tryptophan are slightly better (Table 5).

Table 5. Amino acid composition of protein from ryegrass, red clover and Lucerne extracted in 2017 compared with soybean meal.

Green biomass	Ryegrass	Red clover	Lucerne	Soybean meal
Precipitation	Fermentation	Fermentation	Fermentation	
	Indispensable amino acids, g/100 g crude protein			
Lys	5.79	5.93	5.82	5.98
Met	2.07	1.76	1.94	1.31
Cys	0.74	0.81	0.81	1.43
Thr	4.45	4.43	4.46	3.92
Trp	2.17	2.05	2.17	1.36
Ile	4.91	5.05	5.22	4.91
Leu	8.18	8.36	8.57	7.68
His	2.18	2.36	2.45	2.61
Phe	5.48	5.39	5.76	5.06
Val	6.06	6.18	6.23	5.19
Arg	5.71	5.66	5.65	7.23
	Dispensable amino acids, g/100 g crude protein			
Ala	6.31	5.60	6.16	4.34
Asp	8.81	9.38	9.29	11.36
Glu	9.99	9.93	9.93	17.78
Gly	5.25	5.00	5.22	4.22
Ser	4.26	4.13	4.26	4.97
Tyr	3.79	4.11	4.04	3.69

In Figure 4, the degree of protein hydrolysis determined with the pH-stat method is shown for protein from ryegrass, red clover, lucerne, and soybean meal. The maximum degree of hydrolysis was the highest for ryegrass protein, lowest for lucerne, and intermediate for red clover and soybean meal. The rate of hydrolysis, representing the initial slope of the curve was relatively high for red clover and soybean meal. Combined results from Figures 3 and 4 suggest that without enzyme treatment, the maximum degree of hydrolysis is relatively high for ryegrass protein compared to the other green protein sources. The total and fibrous carbohydrate fraction seems to affect the rate of hydrolysis rather than the maximum degree of hydrolysis. The consequences of these differences for in vivo nutrient digestibility are reported in Deliverable 1.4.

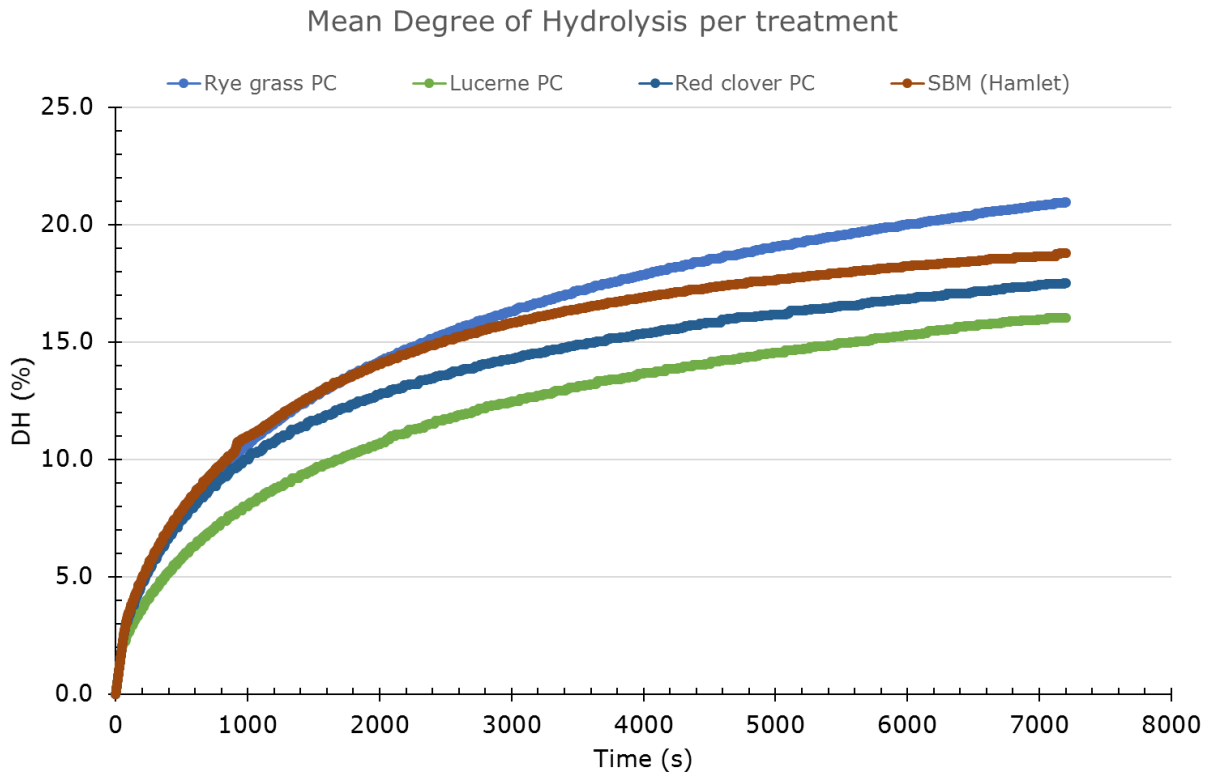


Figure 4. Degree of hydrolysis determined with the pH-stat method of protein from ryegrass, red clover, lucerne, and soybean meal.

3.3.3 Products obtained in 2018

It was clear from the results obtained from the pilot plant for 2016 and 2017 that we were challenged by a high ash content and relatively low concentration of protein in the concentrates obtained from the processing of green biomass. Although the pilot plant was equipped with a sand trap, which removes heavier impurities, it became clear that very fine particles adhered to the protein. The harvesting procedure was therefore changed from 2017 to 2018 in two ways: the direct harvesting and transportation to the loading wagon without contact with the ground and without mulching by a ZeroGrazer (http://zerograzer.ie/) was implemented and the protein from the screw-pressed juice was precipitated by heat (80 °C) rather than by fermentation. As indicated in Table 6, this resulted in a lower ash content and a higher protein content in the protein concentrate (Batches 3 and 4). Furthermore, the ash content also seemed to be reduced by steam precipitation, but this requires further investigations. The amino acid composition of one of the batches produced in 2018 has been completed and showed similar results as those produced in 2016 and 2017 (Tables 2 and 5). The remaining batches are awaiting analysis

Table 6. Ash and protein content of protein concentrate from a grass-clover mixture harvested in 2018 precipitated by fermentation and by steam.

Batch #	Wet amount, kg	Precipitation	Ash, % of DM	Protein, % of DM
Batch 1	168	Fermentation	14.6	38.4
Batch 2	229	Fermentation	18.3	43.0
Batch 3	694	Steam	7.5	49.4
Batch 4	386	Steam	10.2	54.2
Batch 5	39	Fermentation	12.1	38.4

The protein concentrates produced in 2018 were not used in in vivo experiments in the Feed-a-Gene project, but used in other national projects with organic egg layers (GreenEggs) and in a growth experiment with organic pigs (SuperGrassPork). Preliminary results from the experiment with pigs indicate a better growth of pigs fed diets with steam-precipitated protein from green biomass included at levels of 0, 5, 10, or 15% of the diet, compared with a diet based on organic soybean cake, wheat, and faba beans.

3.4 Removal of antinutritional compounds from green protein

Anti-nutritional components is a collective name for components that limit the use and/or decrease the nutritional value of a feed. These components can be part of the feed in its original form, as is the case for grass and clover prior to extraction of protein, or they can be formed during processing (e.g., during the extraction procedure or drying of the protein concentrate).

In a preliminary study using green protein paste precipitated by lactic acid fermentation from the 2016 harvest, the applicability of green protein paste as a raw material in an enzymatic co-processing with soy was studied by Hamlet Protein. It was observed that the green protein paste possesses compounds that contribute to the formation of Maillard compounds during the subsequent drying, resulting in a reduction of lysine availability. This undesired effect was less pronounced when heat-precipitated green protein paste from the 2018 harvest was used, but the availability of lysine remained a matter of concern.

The main anti-nutritional components identified in green protein paste are reaction products from the action of polyphenol oxidase on the macerated biomass. The reaction starts when the grass is cut, but it increases when the biomass is physically separated in the screw press into green juice and pulp, and left to incubate before precipitation. During this incubation period, regardless of whether this is done by lactic acid fermentation of 24 hours or merely a collection of material for 1 hour before heat precipitation, the polyphenol oxidase has access to the substrate due to the physical treatment of the biomass, which induces cross-linking of components that decrease protein digestibility. Specifically, the side chain of cysteine can act as a nucleophile in the oxidation reaction. To counteract this effect by polyphenol oxidase, the protein extraction system would need a re-design to accommodate immediate heat treatment after the physical treatment.

The bioprocessing at Hamlet Protein removed approximately 40% of the measured minerals from the protein fractions (Figure 5). However, a large proportion of the ash content is not accounted for in the minerals analysis and this is most likely silica/sand, and total ash content was therefore only reduced from 11.8 to 10.1**%** of dry matter. The silica/sand levels need to be dealt with in the optimisation of the green biomass processing, both during harvesting (e.g., to avoid molehills) and in the processing technology where more efficient sand filters or eventual centrifugation of green juice prior to precipitation could be considered.

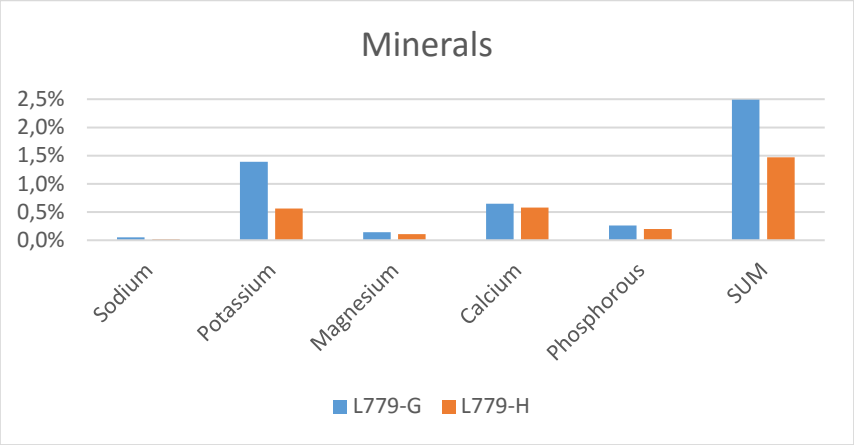


Figure 5. Sodium, potassium, magnesium, calcium, phosphorous, and total minerals (% of DM) before (L779-G) and after (L779-H) removal of antinutritional compounds.

Non-protein nitrogen (NPN) levels of the green protein are high in green protein paste precipitated by lactic acid fermentation or by heat. However, the heat-precipitated product is slightly better with ~6% NPN of crude protein, compared with the lactic acid fermented product with ~9% NPN of crude protein.

The fibres can influence the nutritional value of the green protein if the protein is bound to the cell walls in the fibre matrix that make up a large proportion of the green protein concentrate (Tables 1 and 4). However, the fibres in green biomass are very recalcitrant and resistant to enzymatic hydrolysis by commercially available enzymes. Further research is needed on this topic for optimal digestion of green protein. Furthermore, some enzymes show reduced activity in the presence of green protein indicating the presence of enzyme inhibitors in green protein.

4. Conclusions

The activities in task 1.2 were concentrated on optimizing the extraction conditions for protein of the pilot plant at Aarhus University. The process involved separation of the green biomass into a liquid stream composed of soluble proteins, carbohydrates and minerals, and a fibre-rich solid stream that contains the majority of cell walls and insoluble proteins.

The protein content of the protein concentrates produced from ryegrass and red clover extracted by single pressing without and with the use of cell wall degrading enzymes in 2016 was around 33%. For ryegrass extracted without and with the use of cell wall degrading

enzymes, the protein content of the protein concentrate was more than 3 times higher than in the pulp. However, the use of cell wall degrading enzymes did not increase the protein content of the protein concentrates, but the carbohydrate content of the protein concentrate was slightly lower.

The pilot plant was upgraded in 2017 enabling double pressing and the protein content of the protein concentrates produced from ryegrass, red clover, and lucerne was slightly higher than in 2016 (33.0 to 37.8%). However, the protein content of the protein concentrates produced in 2017, like in 2016, was significantly lower than that of soybean meal. The amino acid composition of the protein concentrates from green biomasses processed in 2016 and 2017 were similar to that of soybean meal.

All protein concentrates produced in 2016 and 2017 had a relatively high ash content (13.5 to 30.5%) but implementation of direct harvesting and collection of the green biomass and the use of steam, rather than spontaneous or lactic acid fermentation for precipitation, increased the protein content of the protein concentrates produced in 2018 to a level (49.4 to 54.2%), which is similar to that of soybean meal.

The in vitro digestibility (rate and extent) of the protein concentrates produced in 2016 and 2017 were similar to that of a good quality soybean meal. The maximum degree of hydrolysis without enzyme treatment was highest for extracted protein from ryegrass. The rate of hydrolysis seems to be related to the level and composition of the carbohydrate fraction.

Protein from green biomass precipitated by acid fermentation is not suitable for the bioprocessing technology used at Hamlet Protein, but it was possible to obtain some modest improvements for steam precipitated green protein. However, the ash content, even in steam precipitated green protein, is still a concern and combining green protein with soybean meal is limited due to the presence of yet to be identified enzyme inhibitors in green protein.

Taken as a whole, there is still a way to go before protein from green biomass can be considered comparable to soybean, meal but progress has been made during the course of the Feed-a-Gene project.

5. Annexes

Annex 1: Pictures of the machineries used in the pilot plant processing of green biomass.

Annex 2: Carbohydrate composition and Klason lignin of protein concentrate from ryegrass and red clover extracted without and with the use of cell wall degrading enzymes.

Annex 3: Carbohydrate composition and Klason lignin of the pulp from ryegrass and red clover after extraction of protein without and with the use of cell wall degrading enzymes.

Annex 4: Carbohydrate composition and Klason lignin of protein concentrate from ryegrass, red clover, lucerne, and soybean meal.

Annex 5: Summary of pH-stat results for the degree of hydrolysis of green protein for the ryegrass and red clover harvest of 2016 without and with the use of cell wall degrading enzymes, and for the ryegrass, red clover, and lucerne harvest of 2017, and soybean meal.

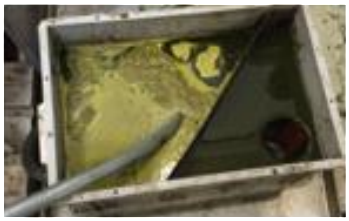
Annex 1. Machineries used in the pilot plant processing of green biomass.

Biomass input system

Shredder

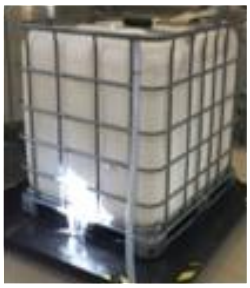
Screwpress - open

Screwpress



Sand-trap

Tank



Fermentation tank

Heat exchange

Decanter centrifuge

Annex 2. Carbohydrate composition and Klason lignin of protein concentrate from ryegrass and red clover extracted without and with the use of cell wall degrading enzymes.

Green biomass	Ryegrass		Red clover	
Enzymes	No	Yes	No	Yes
Precipitation	Heat	Heat	Fermentation	Fermentation
Dry matter, %	98.6	98.2	97.9	96.3
	Values, % of dry matter			
Total-CHO[1]	16.0	8.7	18.0	16.1
Total sugars	0.6	0.1	1.4	0.8
Glucose	0.1	<0.1	0.2	0.2
Fructose	0.2	0.0	0.7	0.3
Sucrose	0.3	0.1	0.5	0.3
Starch	1.3	0.8	1.0	1.0
Fructans	1.6	0.0	2.6	1.1
LMW-NDC	2.8	1.0	2.5	2.8
Rhamnose	<0.1	0.1	0.1	0.2
Fucose	0.0	0.0	0.0	0.0
Arabinose	<0.1	0.1	0.2	0.2
Xylose	0.1	<0.1	0.1	0.2
Mannose	0.1	0.0	0.0	0.0
Galactose	0.3	0.1	0.4	0.3
Glucose	1.7	0.3	1.2	1.3
U.A.	0.5	0.4	0.5	0.7
S-NCP	1.7	2.6	1.7	2.1
Rhamnose	0.1	0.1	0.1	0.1
Fucose	<0.1	<0.1	0.0	0.0
Arabinose	0.2	0.1	0.2	0.2
Xylose	0.1	0.1	0.1	0.1
Mannose	0.2	0.2	0.3	0.2
Galactose	0.3	0.6	0.3	0.5
Glucose	0.3	0.6	0.3	0.5
U.A.	0.6	0.9	0.5	0.5
I-NCP	4.7	1.9	4.7	4.5
Rhamnose	0.1	0.1	0.1	0.1
Fucose	0.1	<0.1	<0.1	<0.1
Arabinose	0.9	0.4	0.9	0.7
Xylose	0.9	0.4	1.1	0.9
Mannose	0.4	0.2	0.4	0.3
Galactose	0.9	0.3	0.9	0.9
Glucose	1.1	0.4	1.2	1.2
U.A.	0.3	0.2	0.1	0.3
Cellulose	3.2	2.3	4.1	3.9
I-NSP	7.9	4.2	8.8	8.4
Total NSP	9.6	6.8	10.4	10.5
Total NDC[2]	14.0	7.8	15.5	14.3
Klason lignin	17.3	17.7	16.0	15.0
Dietary fibre[3]	31.3	25.5	31.5	29.3

Total-CHO, total carbohydrates; LMW-NDC, low-molecular weight non-digestible carbohydrates; U.A., uronic acids; S-NCP, soluble non-cellulosic polysaccharides; I-NCP, insoluble non-cellulosic polysaccharides; I-NSP, insoluble non-starch polysaccharides; Total NDC, total non-digestible carbohydrates.

[1]Total CHO = sugars+starch+fructans+LMW-NDC+S-NSP+I-NSP+cellulose.

[2] Total NDC = fructans+LMW-NDC+S-NSP+I-NCP+cellulose.

[3] Dietary fibre = Total NDC + Klason lignin.

Annex 3. Carbohydrate composition and Klason lignin of the pulp from ryegrass and red clover after extraction of protein without and with the use of cell wall degrading enzymes.

Green biomass	Ryegrass		Red clover	
Enzymes	No	Yes	No	Yes
Precipitation	Heat	Heat	Fermentation	Fermentation
Dry matter, %	98.6	98.2	97.9	96.3
	Values, % of dry matter			
Total-CHO[1]	61.6	62.5	51.1	53.5
Total sugars	1.0	1.3	2.5	1.8
Glucose	0.1	0.1	1.6	1.1
Fructose	0.6	0.8	0.6	0.5
Sucrose	0.3	0.4	0.3	0.2
Starch	0.7	0.9	0.2	0.0
Fructans	2.8	3.4	0.0	0.0
LMW-NDC	4.5	5.1	6.4	7.1
Rhamnose	0.1	0.0	0.0	0.0
Fucose	0.0	0.0	0.0	0.0
Arabinose	0.2	0.3	0.3	0.4
Xylose	1.4	1.8	0.8	1.0
Mannose	0.3	0.3	0.4	0.5
Galactose	0.1	0.1	0.6	0.6
Glucose	1.3	1.5	2.4	2.1
U.A.	1.1	1.0	2.0	2.4
Soluble-NCP	4.2	2.5	8.0	8.0
Rhamnose	0.1	0.1	0.3	0.2
Fucose	0.0	0.0	0.0	0.0
Arabinose	0.4	0.3	1.0	1.0
Xylose	0.2	0.0	0.0	0.0
Mannose	0.3	0.4	0.1	0.0
Galactose	0.4	0.4	0.8	0.8
Glucose	1.5	0.5	0.1	0.1
U.A.	1.3	1.2	6.1	6.0
Insoluble-NCP	21.8	23.6	13.2	13.9
Rhamnose	0.1	0.1	0.2	0.3
Fucose	0.1	0.1	0.1	0.1
Arabinose	3.5	3.7	1.6	1.6
Xylose	11.6	12.7	4.8	5.3
Mannose	0.6	0.5	1.0	1.0
Galactose	1.8	1.8	1.3	1.3
Glucose	1.5	2.4	1.0	1.0
U.A.	2.6	2.4	3.2	3.3
Cellulose	29.4	29.1	20.8	22.7
I-NSP	51.2	52.7	34.0	36.6
Total NSP	55.4	55.3	41.9	44.6
Total NDC[2]	59.9	60.3	48.4	51.7
Klason lignin	11.9	11.3	12.6	15.2
Dietary fibre[3]	71.8	71.6	61.0	66.8

Total-CHO, total carbohydrates; LMW-NDC, low-molecular weight non-digestible carbohydrates; U.A., uronic acids; S-NCP, soluble non-cellulosic polysaccharides; I-NCP, insoluble non-cellulosic polysaccharides; I-NSP, insoluble non-starch polysaccharides; Total NDC, total non-digestible carbohydrates.

[1]Total CHO = sugars+starch+fructans+LMW-NDC+S-NSP+I-NSP+cellulose.

[2] Total NDC = fructans+LMW-NDC+S-NSP+I-NCP+cellulose.

[3] Dietary fibre = Total NDC + Klason lignin.

Annex 4. Carbohydrate composition and Klason lignin of protein concentrate from ryegrass, red clover, lucerne, and soybean meal.

Green biomass	Ryegrass	Red clover	Lucerne	SBM
Precipitation	Fermentation	Fermentation	Fermentation	
Dry matter, %	98.4	99.4	98.8	90.3
	Values, % of dry matter			
Total-CHO[1]	17.51	9.86	18.84	31.92
Total sugars	0.6	0.1	0.1	5.2
Glucose	0.1	<0.1	0.1	0.0
Fructose	0.3	0.0	0.0	0.1
Sucrose	0.1	<0.1	0.0	5.1
Starch	0.7	2.2	4.0	0.4
Fructans	0.5	0.1	0.1	1.5
LMW-NDC	2.4	1.9	3.9	7.8
Rhamnose	0.0	0.0	0.4	0.0
Fucose	0.0	0.0	0.0	0.0
Arabinose	0.1	0.1	0.4	0.2
Xylose	0.1	0.0	0.3	0.1
Mannose	-0.1	-0.1	0.0	0.1
Galactose	0.4	0.3	0.4	3.2
Glucose	1.6	1.1	1.6	4.6
U.A.	0.4	0.4	0.9	0.4
Soluble-NCP	1.8	1.5	3.1	3.9
Rhamnose	0.1	0.0	0.1	0.1
Fucose	0.0	0.0	0.0	0.1
Arabinose	0.3	0.2	0.2	1.0
Xylose	0.1	0.0	0.0	0.2
Mannose	0.2	0.2	0.1	0.2
Galactose	0.3	0.3	0.3	1.4
Glucose	0.4	0.2	0.3	0.0
U.A.	0.5	0.6	1.9	1.1
Insoluble-NCP	4.7	4.7	1.9	4.5
Rhamnose	0.1	0.1	0.1	0.2
Fucose	0.0	0.0	0.0	0.3
Arabinose	1.4	0.4	0.7	2.0
Xylose	1.7	0.3	0.8	1.2
Mannose	0.5	0.2	0.4	0.8
Galactose	0.9	0.6	0.7	3.2
Glucose	1.2	0.8	0.4	0.8
U.A.	0.5	0.2	0.7	1.7
Cellulose	5.2	1.4	3.9	3.1
I-NSP	11.5	4.2	7.7	13.1
Total NSP	13.3	5.7	10.8	17.0
Total NDC[2]	16.2	7.6	14.8	26.3
Klason lignin	19.3	21.1	20.9	4.3
Dietary fibre[3]	35.5	28.7	35.7	30.6

Total-CHO, total carbohydrates; LMW-NDC, low-molecular weight non-digestible carbohydrates; U.A., uronic acids; S-NCP, soluble non-cellulosic polysaccharides; I-NCP, insoluble non-cellulosic polysaccharides; I-NSP, insoluble non-starch polysaccharides; Total NDC, total non-digestible carbohydrates.

[1]Total CHO = sugars+starch+fructans+LMW-NDC+S-NSP+I-NSP+cellulose.

[2] Total NDC = fructans+LMW-NDC+S-NSP+I-NCP+cellulose.

[3] Dietary fibre = Total NDC + Klason lignin.

Annex 5. Summary of pH-stat results for degree of hydrolysis of green protein for the ryegrass and red clover harvest of 2016 without and with the use of cell wall degrading enzymes, and for the ryegrass, red clover, and lucerne harvest of 2017, and soybean meal.

ID	Products	DHmax (%)	k, 10^{-5}	Initial pH
1	Ryegrass without enzyme	20.65^{ab}	4.41^{ab}	4.66^{c}
2	Ryegrass with enzyme	18.66^{ab}	8.31^{c}	4.66^{c}
3	Red clover without enzyme	18.42^{a}	3.65^{ab}	4.97^{d}
4	Red clover mix with enzyme	20.14^{ab}	4.04^{ab}	4.33^{ab}
5	Ryegrass PC	24.09^{c}	3.24^{a}	4.14^{a}
6	Lucerne PC	18.48^{ab}	4.14^{ab}	4.52^{bc}
7	Red clover PC	19.10^{ab}	5.76^{b}	4.19^{a}
8	Soybean meal (Hamlet)	20.81^{b}	5.34^{ab}	6.66^{e}
	SEM	0.77	0.82	0.081
	P-value	0.002	0.014	<0.001

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