# In vitro digestibility of grass and clover species after biorefining- effect of cut.

## Background:

Variations in extraction efficiency and product quality challenge the successful implementation of a commercial biorefining process. Optimizations within the biorefining plants have significantly improved the extraction yield and increased the quality of the produced products, however many of the challenges arise in the field before harvest, where optimizations during processing will only have limited effect. To maximize the utilization of the biorefinery, plants of high quality must be available for the full growth season. The chemical composition and nutritional quality of grasses and legumes vary during the season and are influenced by plant species and growth conditions. In addition, plant maturation is associated with a shift in leaf:stem ratio resulting in declining protein concentration and a subsequent increase in the concentration of less digestible fibre and lignin[1]. Variations in chemical composition affect the ability to extract protein, which will differ between species. A higher extractability has been found in legumes[1, 2]

It is important to identify the extent of the seasonal variations in extractability and quality across plant species with the aim of selecting candidates with consistently high quality and yield.

## Analytical methods:

Plants were obtained from DLF, Store Heddinge where they were harvested in a first and third cut strategy. The plant material was frozen at -20C immediately after harvest and transported to Aarhus University. The plant material was thawed overnight at 4 °C prior to processing in a commercial lab-scale twin-screw press (Angelia 8500S, Angel Juicer) resulting in a fibrous pulp and a green juice. Soluble proteins in the juice were precipitated with 12M phosphoric acid until pH=4. The acidified juice was left overnight at 4°C and subsequently centrifuged for 10 min at 2000 g/ 4°C separating the precipitated protein from the residual brown juice. A subsample of the original plant material and the

precipitated protein were freeze-dried, and dry matter (DM) content was determined on freeze-dried material by drying at 103°C. Nitrogen was analysed by the Dumas procedure.

Nitrogen recovery was calculated from DM-based numbers:

$$Nitrogen\ recovery_{fraction}(\%) = \frac{nitrogen\ content_{fraction} * mass_{produced\ fraction}}{nitrogen\ content_{plant} * mass_{processed\ plant}} * 100\%$$

The quality of the precipitated protein was assessed by in vitro protein digestibility as described [3] and the degree of hydrolysis (DH%) was determined.

**Results and discussion:**

The plant material provided by DLF is listed in Table 1. Different varieties of grass and clover harvested at cut 1 and cut 3 were used. The grasses were a combination of early, intermediate and late varieties with different fertilization regimes (2N/4N) and the clovers were red, white and alsike clover.

| | | |
|---|---|---|
| Hybrid ryegrass | HYRG-4N-Inter | Tetratop |
| Festulolium | LMxFA-- | Hykor |
| Festulolium | LMxFA-- | hipast |
| Festulolium | LMxFA-- | Hemsut |
| Festulolium | LMxFA-- | Mahulena |
| Festulolium | LMxFP-- | Hostyn |
| Festulolium | LMxFP-- | Perseus |
| Perennial ryegrass | PRG-2N-Early | Genesis |
| Perennial ryegrass | PRG-2N-Inter | Nabesna |
| Perennial ryegrass | PRG-2N-Inter | Boyne |
| Perennial ryegrass | PRG-2N-Inter | Fabiola |
| Perennial ryegrass | PRG-2N-late | Zorgue |
| Perennial ryegrass | PRG-4N-Early | Giant |

| | | | |
|---|---|---|---|
| Perennial ryegrass | PRG-4N-inter | Velonit | |
| Perennial ryegrass | PRG-4N-inter | Dexter | |
| Perennial ryegrass | PRG-4N-inter | Ritchie | |
| Perennial ryegrass | PRG-4N-inter | Trintella | |
| Perennial ryegrass | PRG-4N-inter | Garbor | |
| Perennial ryegrass | PRG-4N-inter | Melforce | |
| Perennial ryegrass | PRG-4N-late | Magena | |
| Red clover | RedCl-- | Hammon | |
| Red clover | RedCl-- | Callisto | |
| Tall fescue | TallF-- | Tower | |
| Tall fescue | TallF-- | Bannock | |
| Alsike clover | AlsikeCl-- | Ermo | |
| White clover | WhiteCl-- | Klondike | |
| White clover | WhiteCl-- | Briana | |
| Orchard grasses | CocksF-- | | |
| Timote | Timothy-- | | |

The content of nitrogen in the plants used is shown in figure 1.

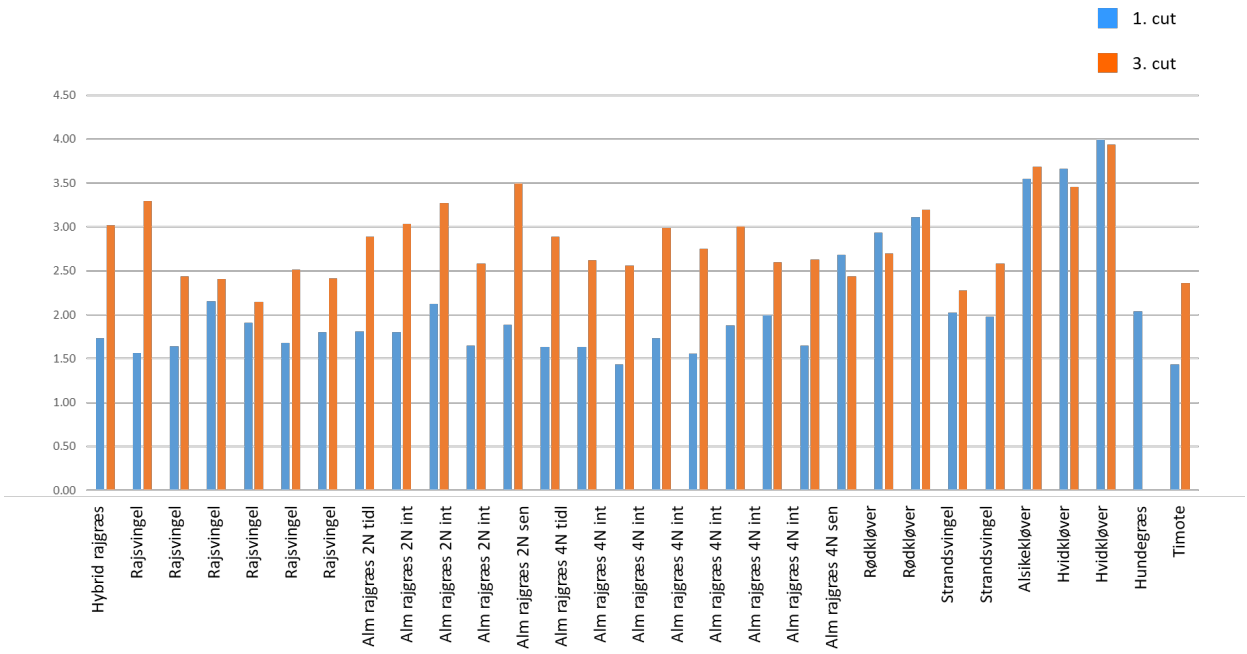


*Figure 1. Nitrogen content (g/100 g DM) in plants harvested at first and third cut. The order of the samples corresponds to the order listed in table 1.*

For all grasses and as expected, N content was found to be higher in the third cut than in the first cut. For the clovers, N values were more similar between the cuts, due to a higher firth cut N content than in the grasses.

The content of nitrogen in the extracted protein is shown in figure 2.

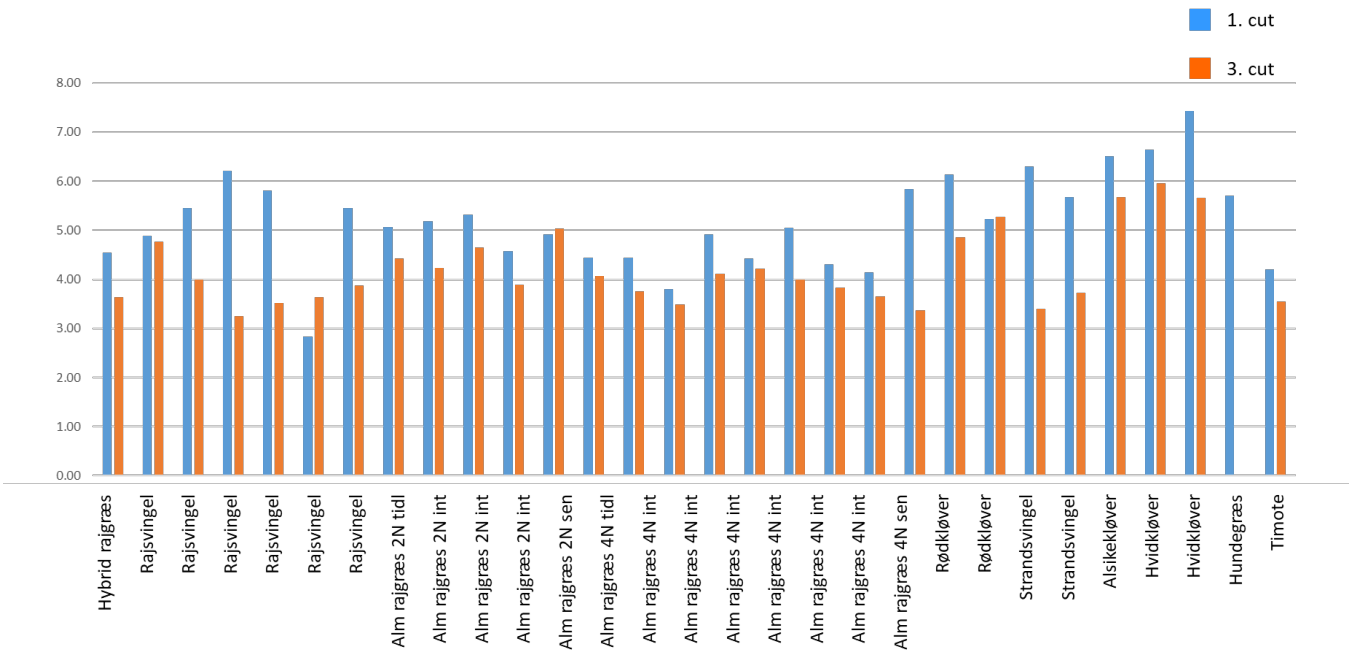


*Figure 2 Nitrogen content (g/100 g DM) in the extracted protein from first and third cut plants. The order of the samples corresponds to the order in Table 1.*

The nitrogen content of protein extracted from first cut plant ranged from 2.84 g/100 g DM in the festulolium variety Hostyn to 7.43 g/100 g DM in protein from the white clover Briana. From the third cut plants, protein was extracted with a nitrogen content ranging from 3.25 g/100 g DM in the festulolium variety Hemsut to 5.96 g/100 g DM in the white clover Klondike. The nitrogen content was generally lower in protein extracted from third cut plants and thus, a high nitrogen content in the plant does not result in a high nitrogen content in the extracted protein. Nitrogen in the plant is both protein-bound and non-protein-bound. The precipitation of soluble proteins in the green juice during the biorefining relies on larger protein structures and smaller non-protein-bound nitrogen components will be distributed to the residual brown juice. The findings in this study could indicate that a larger proportion of the plant nitrogen is found in smaller complexes in third cut plants or that other components such as fibre is co-precipitated to a larger degree from third cut plants thus “diluting” the protein. The phenomenon is most pronounced in grasses.

The sustainability of the biorefining process in terms of economy and environment is conditioned by an efficient utilization of the components in the plant. Presently, much effort is put into optimizing the process regarding maximizing the quality and yield of the extracted protein. The extraction yield, defined as the proportion of plant nitrogen (crude protein) being distributed to the extracted protein is shown in figure 3.

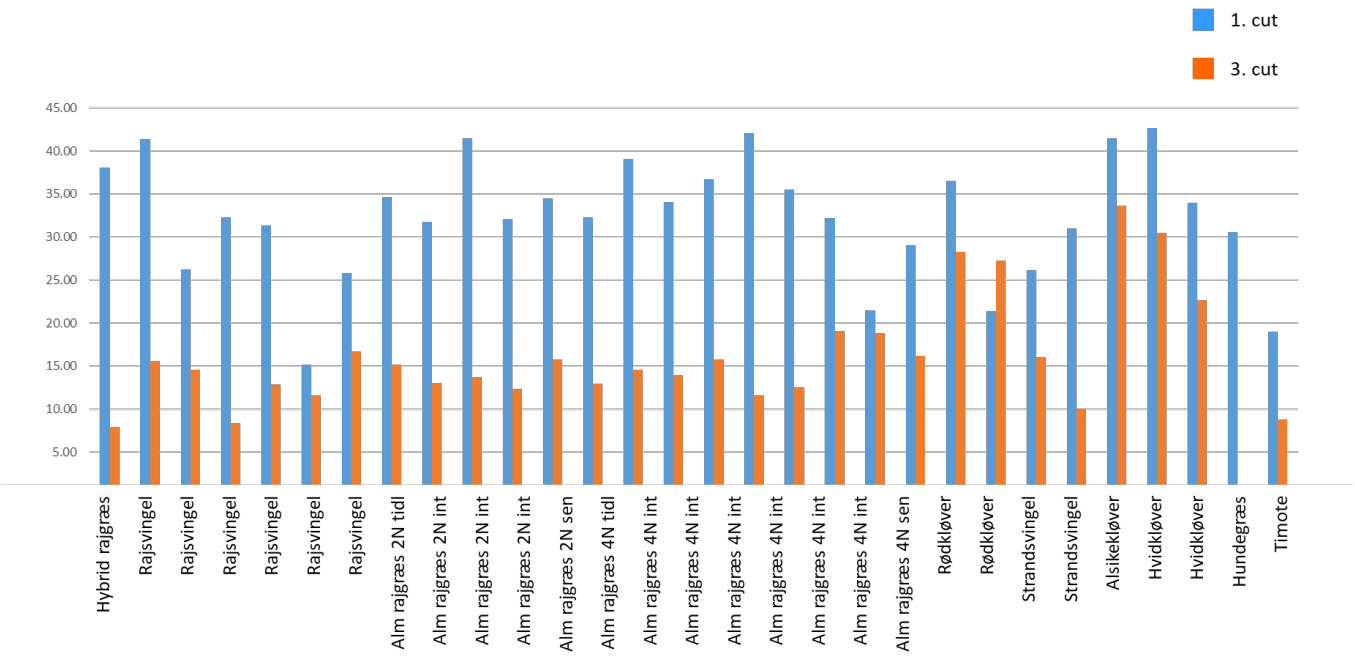


*Figure 3 Extraction yield (proportion of plant protein distributed to the extracted protein) from first and third cut. The order of the samples is found in Table 1.*

From the first cut plants, the N extraction yield ranged from 18.9 % in the perennial ryegrass Melforce to 42.7 % in the white clover Klondike. For third cut plants the yield was 7.9-33.6 %, lowest in the hydrid ryegrass and highest in the alsike clover. Large variations in yields across and within species were detected. The yield was generally higher in third cut clover than in third cut grasses, but a similar pattern was not seen for the first-cut plants. More nitrogen was extracted from the first cut plants than for the third cut for all the plants analysed, except for the red clover Hammon. For some grasses, extraction in the 3rd layer is less than 20% of the extraction in the 1st layer and thus there is really something to be gained by planning a specific utilization of individual layers. Contrary to expectations, there is thus no correlation between a high nitrogen content in the plant and a high degree of extraction. This could be a result of differences in N:protein ratios discussed previously.

It has been speculated whether a low extraction yield could be associated with a high content of Klason lignin. This would not only distribute a larger proportion of the protein to residual press cake but likely also result in a higher degree of Klason lignin precipitated to the protein extract. The content of Klason lignin is high in all protein extracts (15-25% of DM). However, data from the current study does not reveal a higher Klason lignin content in protein extract from plants with a lower yield, nor a higher content in third-cut plant

protein extracts. The content of the non-starch polysaccharides was also analysed and ranged from 21-40% of the DM in the protein extract. The NSP content tended to be higher in protein from the third cut plants. Combined NSP and Klason-lignin constituted approximately 50 % of the DM.

The quality of the extracted protein was assessed in an in vitro digestibility trial, where the digestive tract of the pig was mimicked by using incubation with the relevant digestive enzymes as described[3] (figure 4 and figure 5). The experiment results in a degree of hydrolysis (DH%) reporting the maximum degree of digestion. The DH is an important parameter related to quality.

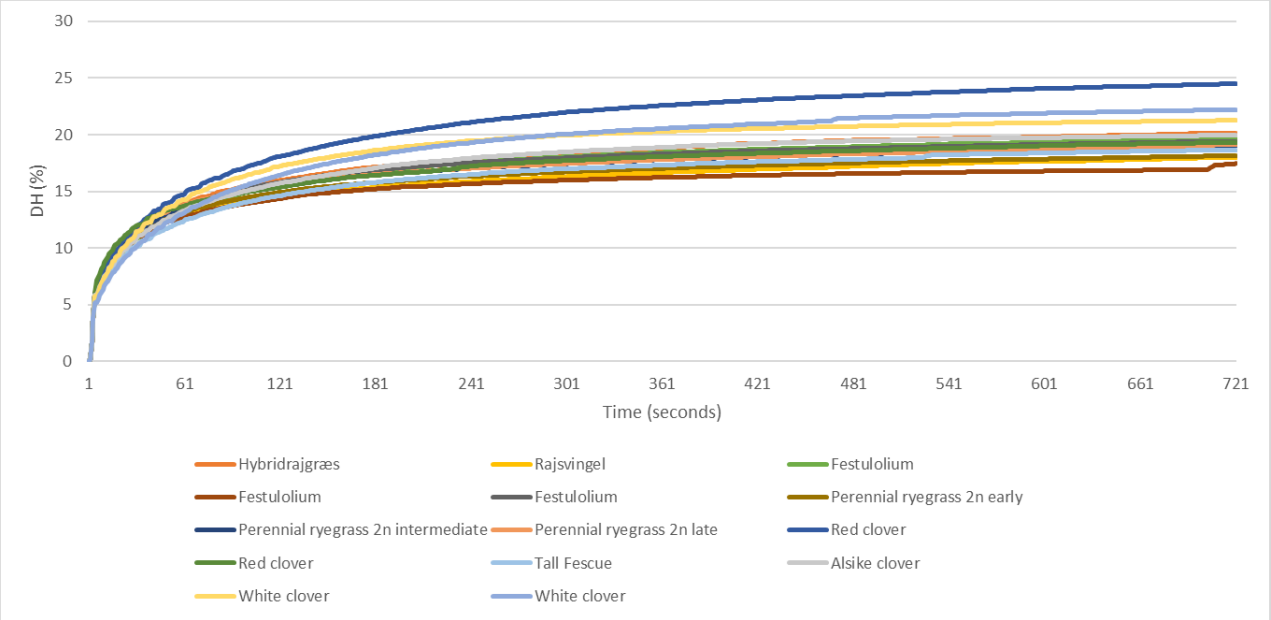


*Figure 4 Degree of hydrolysis (DH%) of protein extracted form first cut plants. For simplicity, only selected samples are included in the figure.*

For protein extracted from first cut plants, no clear effect of plant species/variety was found, as exemplified by figure 4. Some varieties of the clovers had a high DH however, others had low DH. Similar results were seen for the grasses. The highest DH was found in red clover Hammon and white clover Brianna, whereas the lowest was seen in Festulolum Mahutera. The results on protein extracted from third cut plants indicates that the protein in general had a lower DH% than from first cut (figure 5). The alsike clover had the highest DH%, whereas orchard grass had the lowest. DH% in the red clover Hammon was high, like the results from first cut.

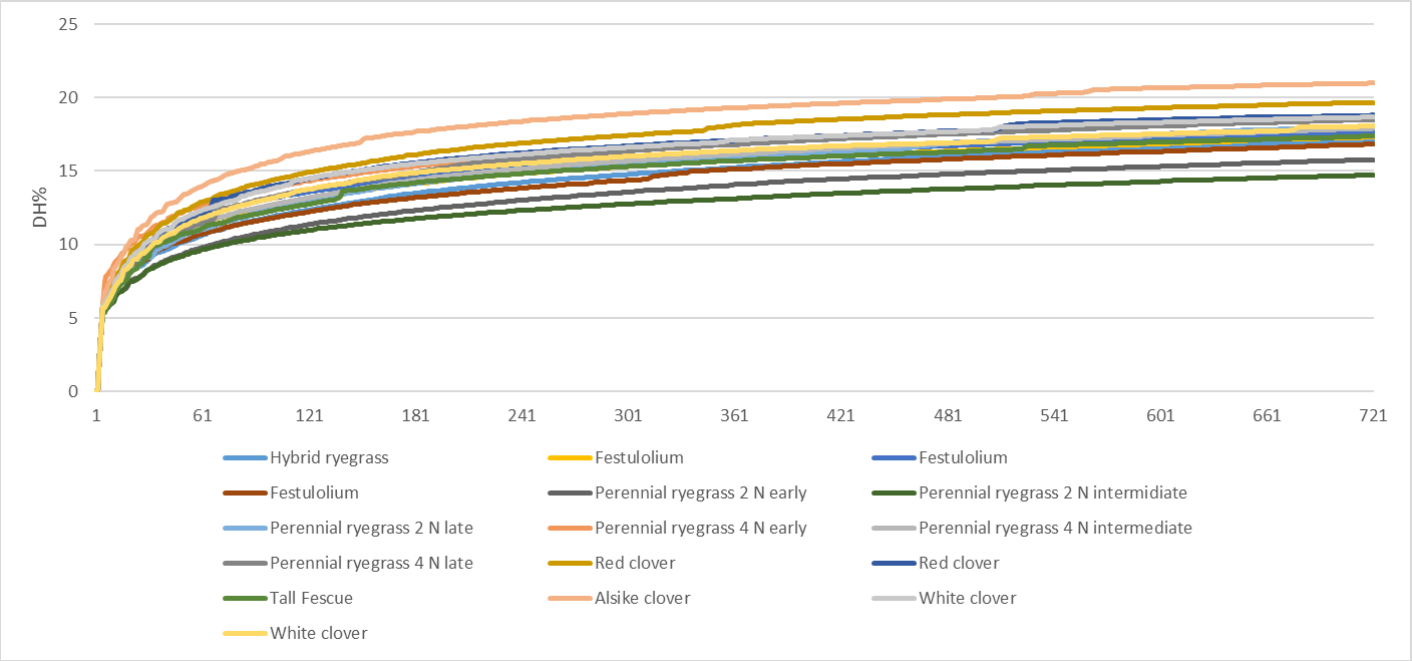


*Figure 5 Degree of hydrolysis (DH%) of protein extracted from third cut plants. For simplicity, only selected samples are included in the figure.*

The degree of digestion will be affected by the associations of nitrogen. Nitrogen bound as free, soluble protein will likely result in a higher DH because the enzymes will have easy access to the nitrogen, whereas nitrogen bound in complex structures such as fibre and other macromolecules will be more inaccessible. To elucidate the underlying cause of the result, the proportion of nitrogen associated to the Klason lignin fraction was analysed. For all samples, a large proportion of N was found in Klason lignin, up to 15-20% for some of the samples. There may be a small pattern of more N bound to Klason lignin in protein from third cut samples, potentially explaining the lower DH%. At single plant level, no correspondence between a high degree of Klason lignin-bound N and low DH was seen. Despite a high DH in alsike and red clover, these plants also had the high proportion of N associated to Klason lignin.

Conclusion:

Protein was extracted on a laboratory scale from grass and clover varieties harvested in the 1st and 3rd harvest. The tests showed that approx. half of the plant's nitrogen can be extracted in a protein product, but at the same time large differences are seen across species and between different varieties within the same species, which is unrelated to the nitrogen content of the plant. For some grasses, the yield is as low as 10% of the plant's nitrogen, which will make economically profitable biorefining difficult. A significantly greater extraction was seen in plants from first cut than from third cut plants.

The quality, i.e., in vitro digestibility, was generally higher in protein from first cut plants, with no clear pattern between species and varieties. Previous experiments have shown small variations in the amino acid composition of the extracted protein, where a high content of legumes in grassland crops increases the lysine content of the extracted protein, while the grasses contribute higher methionine. Since red clover dominates in mixtures when you get to the warmer periods in later seasons, it is assumed that the lower yield and digestibility in later seasons will also be followed by a higher lysine content in the extracted protein. To achieve the most precise feed formulations, it will be necessary to carry out several batch analyses, to ensure a correct supply of amino acids and to avoid an oversupply of protein to the pigs. In this way, the economic and environmental potential of grass protein is best utilised.

1. Stødkilde L, Lashkari S, Eriksen J and Jensen SK, Enhancing protein recovery in green biorefineries through selection of plant species and time of harvest. *Animal Feed Science and Technology* **278**:115016 (2021).
2. Damborg VK, Jensen SK, Weisbjerg MR, Adamsen AP and Stødkilde L, Screw-pressed fractions from green forages as animal feed: Chemical composition and mass balances. *Animal Feed Science and Technology* **261**:114401 (2020).
3. Ingerslev AK, Rasmussen L, Zhou P, Nørgaard JV, Theil PK, Jensen SK and Lærke HN, Effects of dairy and plant protein on growth and growth biomarkers in a piglet model. *Food Funct* **12**:11625-11640 (2021).