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Review article

Production of leaf protein concentrates in green biorefineries as alternative feed for monogastric animals

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ABSTRACT

Photosynthetic active green leaves from grasses, legumes and other plants contain in general high amounts of proteins, which can be utilized by ruminants or in many cases are wasted. Extraction of proteins from leaves represents an attractive solution to the increasing demand for protein-rich feed for monogastric animals, while decreasing the dependency on soybean imports. Furthermore, there is an increasing demand for organic products produced without the use of chemical fertilizers, pesticides or other artificial chemicals. Leaf proteins extracted from organically grown crops, especially legumes could be attractive as animal feed. Even though a lot of research was carried out in this field previously, recent technological development and biotechnological advances together with the increasing demand may facilitate the industrial implementation of leaf protein extraction processes nowadays. This review focuses on the concept of leaf protein and its history, the different methods for the extraction of proteins from leaves, and the nutritional value of the leaf protein concentrates for feeding monogastric animals. Furthermore, the review focuses on the potential integration of leaf protein extraction within green biorefineries, where freshly harvested leafy plant material is processed into a broad range of products, including feed, food, chemicals, materials and biofuels. The integration of production of leaf protein concentrates within green biorefineries will encourage the establishment of production facilities, also focusing on utilization of the different residue streams. Thereby such green biorefineries can contribute to the development of more self-sufficient and sustainable agricultural systems in Europe.

1. The need for alternative protein feeds

The world's livestock sector is growing rapidly in order to meet the high demand for meat and dairy products, which has increased 1.5-fold over a 50 years period (i.e. 1960–2010) (Godfray et al., 2010; McMichael et al., 2007). In 2018, the European livestock population was approximately 333 million head (excluding poultry), and included 148 million pigs (EUROSTAT, 2020). Denmark is the fourth largest pig producer in the EU with 8.5 % of the total pigs, just after Spain, Germany and France (EUROSTAT, 2020). Definitely, the EU has a strong self-sufficiency in terms of protein production for human consumption since most of the available meat in EU is produced within the EU; however, the EU is greatly dependent on imported protein-rich feed materials, mainly soybeans and soybean meal (de Visser et al., 2014).

Abbreviations: DM, dry matter; GMO, genetically modified organism; LPC, leaf protein concentrate; Rubisco, ribulose-15-bisphosphate carboxylase/oxygenase; TCA, trichloroacetic acid

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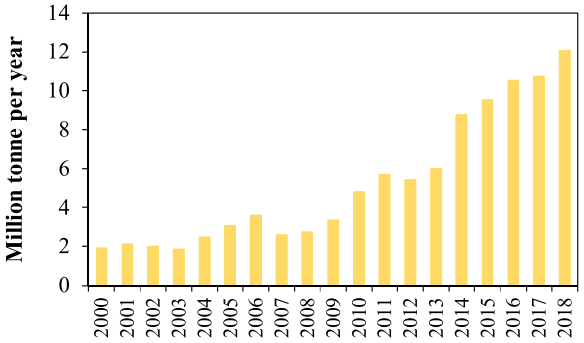

Fig. 1. Soybean production in Europe (FAOSTAT, 2020).

Soybean imports in the EU accounted for 15.3 million tonne per year (average for the period 2013–2017), with the Netherlands, Germany and Spain being the top three European importers (FAOSTAT, 2020). The soybean cake (or soybean meal) import in the EU was almost twice this amount averaging 25.3 million tonne per year for the period 2013–2017 (FAOSTAT, 2020) mostly because of the large dependency on soybean meal for monogastric animals (de Visser et al., 2014). In general, the production of soybeans is increasing worldwide to meet the strong demand for soybean products, markedly led by China. The production of soybeans is also increasing in Europe (Fig. 1) in order to diminish the dependency on imports and the likely risk for volatile prices. In fact, the soybean production in Europe increased 6.3-fold from 2000 to 2018. The greatest increase in the European soybean production occurred in Ukraine with a 5.5-fold increase during a 10-year period (2008–2018), and in Russia with a 5.4 increase during a 10-year period (2008–2018) (FAOSTAT, 2020). Nevertheless, soybean production in Europe represents only 3.5 % of the world's soybean production, which was almost 349 million tonne in 2018 and was led by US and Brazil (FAOSTAT, 2020). However, soybean production is related with significant environmental impacts. For instance, oversea transportation for export purposes and more importantly, long distance road transportation within the production country, such as in Brazil contribute significantly to the greenhouse gas emissions associated with soybean production (Castanheira and Freire, 2013; Prudêncio da Silva et al., 2010). Furthermore, most soybeans grown in North and South America are genetically modified, which limits their use for customers that seek non-GMO and organic products. The fact that soybean cultivation and livestock production in Europe take place in different geographical locations also disrupts the cycle of nutrients i.e. the manure produced from livestock fed with soybeans is not available as fertilizer for the land cultivation of soybeans triggering serious soil erosion problems (Taelman et al., 2015). Moreover, the expansion of soybean cultivation areas provokes deforestation and loss of natural areas and ecosystems as happened with a portion of the Brazilian Amazonas utilized for the massive development of soybean infrastructures (Fearnside, 2001).

The interest on organic farming is growing at a fast pace globally as well, but still represents a small percentage of total crop, livestock, and poultry production. About 71.5 million hectares were managed organically worldwide by the end of 2018, representing a 2.9 % increase compared to 2017 (IFOAM, 2020). In Europe, the organic agricultural land accounted for 15.6 million hectares in 2018, representing a 3.4-fold increase from 2000 (Fig. 2) (FiBL Statistics, 2020). The proportion of organic farmland relative to conventional farmland is also increasing steadily in Europe (Fig. 2) but represented only 3.1 % of the total farmland area in 2018 (FiBL Statistics, 2020). In the EU, the organic area share accounted for 7.7 % of the total farmland area, with Spain, France, Italy and Germany holding about 50 % of the total organic area (EUROSTAT, 2020; FiBL Statistics, 2020). In Denmark, the organic area represented 9.8 % of the total farmland area and accounted for around 257 thousand hectares in 2018 (FiBL Statistics, 2020).

The organic livestock sector is also growing in order to meet the increasing demand for organic products. In the EU, the organic poultry sector is developing faster than the livestock sector, and poultry production has skyrocketed during the last two decades (Fig. 3) (EUROSTAT, 2020; Rossi, 2016). In 2018, the organic live poultry in the EU accounted for 37.2 million head of which 34 % were organic laying hens. France is the leading organic poultry producer in the EU, with 34 % of the total organic production in 2016

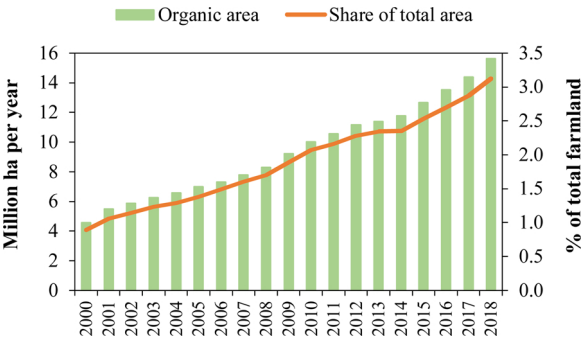

Fig. 2. Total organic farmland area in Europe (FiBL Statistics, 2020).

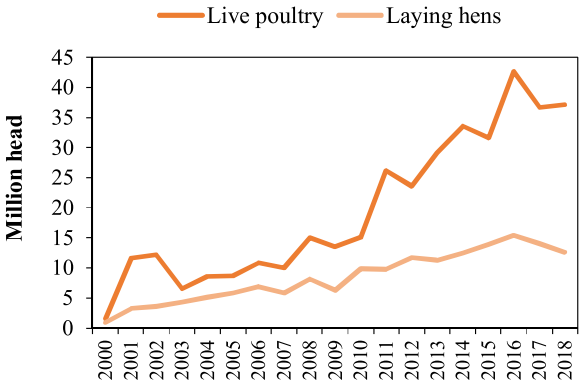

Fig. 3. Total organic live poultry (including laying hens and broilers) and organic laying hens in the European Union (EUROSTAT, 2020; Rossi, 2016).

while Denmark accounted for 6 % of the organic poultry production in the EU (Fig. 4A) (EUROSTAT, 2020). In contrast, the organic pig sector is still very limited in the EU compared to the total pig market and to other livestock, partly because of the difficulties for providing organic animal feed (Rossi, 2016). Denmark is the main organic pig producer in the EU, with 27 % of the total organic pig production in 2016 (Fig. 4B) (EUROSTAT, 2020). One of the main challenges in organic livestock production is the quality and availability of organic feed (van de Weerd et al., 2009). According to the Council Regulation (EC), 2007, which establishes the legislation for the organic production and labelling of organic products in the EU, organic livestock should be fed with 100 % organic feed from the same farm or from farms in the same region; and the use of synthetic amino acids or growth promoters is prohibited (European Commission, no date). However, a maximum percentage of 5 % non-organic feed was authorized in livestock feeding in case farmers were not able to feed exclusively organic until December 2011 (Commission Regulation (EC), 2008), that was extended until December 2017 (Commission Implementing Regulation (EU), 2014) and later until December 2018 (Commission Implementing Regulation (EU), 2017). However, as the organic protein supply is not sufficiently available to meet the demand, it is allowed to use a limited proportion of non-organic protein feed for poultry and pigs until December 2020 (Commission Implementing Regulation (EU) of 22.10.2018). Accordingly, the utilization of soybean meal, which is the most common protein source in conventional feeding of monogastric animals, is only allowed in organic livestock feeding, if produced from organic soybeans without the use of organic solvents. The reason for soybeans and especially soybean meal being the most important protein source lies in the superior amino acid profile that currently makes soybean meal the plant protein product that best matches animal requirements. The production of organic soybeans, which can be utilized for the production of organic soybean meal, represents less than 0.1 % of the total worldwide production (Hartman et al., 2016). Therefore, the availability of organic soybeans and organic soybean meal for animal feeding is limited.

In this context, novel protein-rich sources are required for livestock feeding in Europe, especially for monogastric animals and suitable for organic farming. Furthermore, more sustainable farming systems should be developed based on the utilization of locally produced feeds (Santamaría-Fernández et al., 2017). Grain legumes (or dry pulses) like chickpeas, faba beans, lupines or peas could

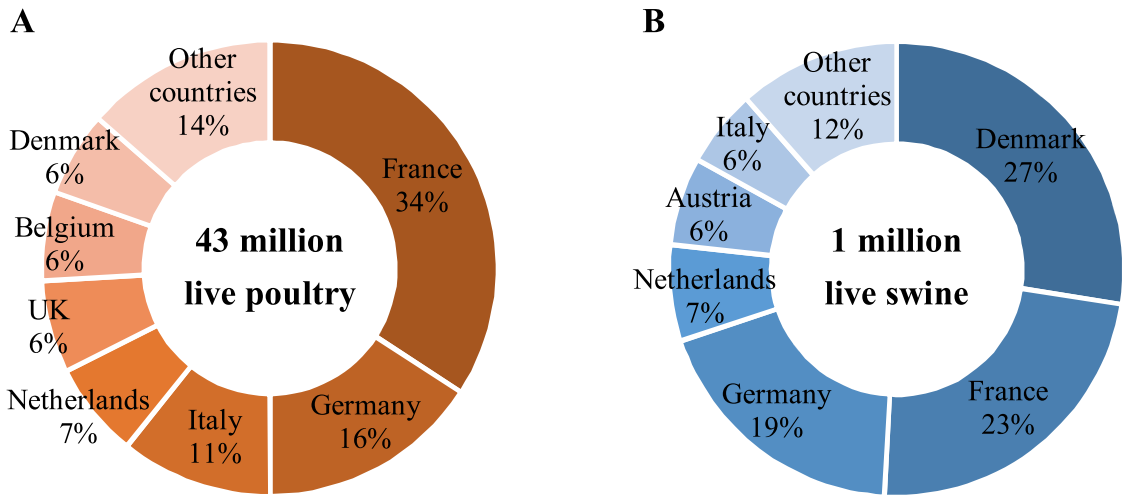

Fig. 4. Percentage of the total 43 million live organic poultry (including laying hens and broilers) (A) and percentage of the total 1 million live organic swine (including breeding sows, fattening pigs and other pigs) (B) by country in 2016 in the European Union (EUROSTAT, 2020).

be grown as an alternative to soybean imports as they are a valuable source of dietary protein for both human and animal feeding (Boye et al., 2010; Schumacher et al., 2011). Nevertheless, grain legume proteins are mostly deficient in tryptophan and in sulphur amino acids i.e. methionine and cysteine that are highly important in animal feeding (Boye et al., 2010; Schumacher et al., 2011). Grain legumes contain naturally occurring anti-nutritional factors such as protease inhibitors, lectins and tannins that might affect the protein digestibility and the amino acid availability (Gatel, 1994). Although, the presence of anti-nutritional factors could be eliminated or inactivated by heating and processing (Boye et al., 2010), the lack of sulphur amino acids should be balanced with other protein sources to meet the animal requirements. Alternatively, protein extraction from plant leaves may be a suitable solution for providing protein-rich feeds for monogastric animals, especially if the leaf protein extracts have amino acid profiles similar to soybeans (Santamaría-Fernández et al., 2017). The integration of protein extraction within green biorefineries could positively influence the overall economics making protein extraction more profitable (Dale et al., 2009) and could be attractive for providing more sustainable protein sources than soybeans for livestock production in Europe (Parajuli et al., 2015). Green biorefineries represent integrated systems for exploitation of green crops *i.e.* grasses, legumes and catch crops or the green part of crops (e.g. beets and carrots), which can be utilized fresh or ensiled for the production of feed, food, chemicals, materials and biofuels (Kamm et al., 2016). Due to their nitrogen fixing ability, legumes such as clover and alfalfa are valuable forage crops that reduce the need for nitrogen fertilizer, and especially in organic farming, these crops are important for crop rotations, delivering nitrogen to the succeeding crops.

This review focuses on the concept of leaf protein, its history and methods for extraction of protein from leaves, the nutritional value of the leaf protein concentrates (LPC) for feeding monogastric animals, and the potential integration of leaf protein extraction within green biorefineries.

2. The leaf proteins

Leaves are the largest source of proteins in the world (Ellis, 1979; Fiorentini and Galoppini, 1983). Most proteins in plant leaves (about 80 %) are located in the chloroplasts, where about half of the proteins are soluble in the stroma and the other half are part of the thylakoid membranes (Fiorentini and Galoppini, 1983; Tamayo Tenorio et al., 2018). The thylakoid membranes are networks of membranes containing proteins, lipids and pigments (chlorophyll and carotenoids) specialized in the photosynthesis and embedded in the chloroplasts stroma (Ellis, 1977). More than 70 different proteins are involved in the photosynthetic reactions taking place is the thylakoid membranes (Friso et al., 2004). The remaining proteins in plant leaves are mostly located in the cytoplasm (about 20 %) with minor amounts found in the cell nucleus ($1-2$ %) or in the mitochondria (less than 5 %) (Fiorentini and Galoppini, 1983). Around 250–300 different proteins and polypeptides were detected by electrophoresis in green plant extracts (Kromus et al., 2006).

Leaf proteins can be differentiated between insoluble and soluble based on their solubility in water. The insoluble protein fraction is mainly composed of proteins forming the photosynthetic complexes together with lipids and pigments in the thylakoid membranes of the chloroplast (Fiorentini and Galoppini, 1983; Tamayo Tenorio et al., 2018). A small fraction of insoluble proteins can be also found in the cell wall attached to polysaccharides (Tamayo Tenorio et al., 2018). The soluble protein fraction is predominantly Rubisco (Ribulose 1,5-bisphosphate carboxylase/oxygenase), the key enzyme for the fixation of CO_2 during photosynthesis that can represent up to 50 % of the total soluble proteins in the leaves (Fiorentini and Galoppini, 1983; Tamayo Tenorio et al., 2018). Rubisco is a relatively large enzyme with an approximate molecular weight of 550 kDa, composed of eight large subunits (55 kDa) and eight small subunits (15 kDa) and located in the stroma, where it catalyzes the first step of the photosynthetic process. However, Rubisco has a very low catalytic efficiency that plants overcome by synthesizing large Rubisco amounts in the leaves (Nishimura et al., 2008). The remaining soluble proteins in the leaves are enzymes involved in the synthesis of carbohydrates, lipids, proteins and other compounds as well as free amino acids and oligopeptides (Ellis, 1977; Fiorentini and Galoppini, 1983). Therefore, plant leaves represent a vast source of proteins that in many cases are utilized by grazing livestock but potentially could be used for monogastric animal feeding purposes.

3. Extraction of proteins from leaves

The extraction of proteins from leaves is not a new concept since a lot of research was carried out in this field during the 20th century, especially during the Second World War. At that time, the possibility of utilizing leaf proteins as human food appeared as an alternative for providing populations with sufficient protein in the event of food shortages (Kromus et al., 2006; Pirie, 1971). Pirie and colleagues at the Rothamsted Experimental Station (England) carried out highly valuable research in order to develop a large scale process for the extraction of proteins from fresh leaves to be used as food protein in human nutrition (Morrison and Pirie, 1961; Pirie, 1969, 1971, 1987). Nevertheless, leaf protein concentrates were not well-accepted by consumers because of their bitter, grassy flavor and dark green color (Chiesa and Gnansounou, 2011; Edwards et al., 1975). Moreover, the development of new protein concentrates from sources such as soybean, peanut or whey expanded faster and their inclusion in human foods slowed down the ongoing research on leaf protein (Edwards et al., 1975). In the US, research focused on developing a process for the production of a protein-xanthophyll concentrate, so called PRO-XAN intended for poultry feeding coupled with the commercial production of dehydrated alfalfa meal for ruminants feeding (Knuckles et al., 1972; Lazar et al., 1971). Pigments such as xanthophyll and beta-carotene are valuable for poultry and the yolk color of eggs (Arkcoll, 1973). A pilot plant for the production of PRO-XAN from alfalfa residual juice was built (Lazar et al., 1971) and later on, the PRO-XAN process evolved for the fractional production of a chlorophyll-containing green protein concentrate (PRO-XAN II) for monogastric animals and a white protein concentrate (WELPRO) suited as food grade protein product (Edwards et al., 1975). In the last couple of decades, the interest on the protein extraction from plant

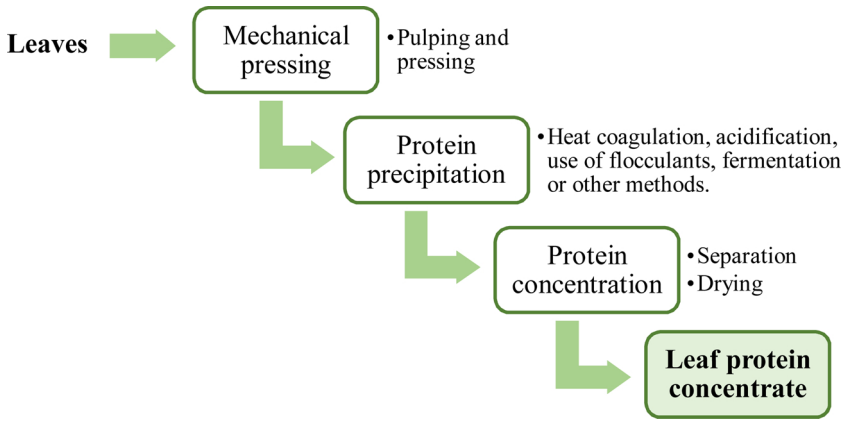

Fig. 5. Basic unit operations for the extraction of proteins from leaves.

leaves has been rekindled probably driven by factors such as the need for alternative protein sources with food and feed applications, the industrialization and development of biotechnologies, and the concept of biorefineries for producing wide range of commodities as alternative to oil refineries.

The extraction of proteins from leaves involves an initial mechanical pressing of the fresh material so leaf proteins are released into the pressed plant juice; followed by protein precipitation and protein concentration into the so-called LPC (Fig. 5).

3.1. Mechanical pressing

Mechanical pressing is carried out to separate the leaf proteins from the cell wall so that a protein rich juice is squeezed out from the fibers (Bals et al., 2012). Traditionally, fresh leaves were firstly pulped with different mills or rollers to break up the cells and release the cellular components including soluble proteins and chloroplasts; then, the juice was expressed from the fibrous pulp (or press cake) by pressing (Bals et al., 2012). Being that pulping and pressing could not be performed in a single unit at large scale due to technological limitations, Pirie (1971, 1987) carried out an extensive research to develop an economic process for the plant juice extraction. For instance, several pulpers alternative to hammer mills were developed to continuously pulp the crops (Pirie, 1971). Further, Pirie (1987) focused on developing presses to press out most of the extractable juice by applying pressure very efficiently. Besides, a twin-screw press was utilized in the PRO-XAN process for pulping and pressing alfalfa in one single unit operation (Knuckles et al., 1972). The twin-screw press consisted of two counter-rotating screws and was able to squeeze out 65 % of the fresh weight from chopped alfalfa into the juice with a 40 % protein recovery (Knuckles et al., 1972). More recently, the mechanical pressing is commonly performed in screw presses, which combine the application of pressure with additional maceration of the cell walls resulting in approx. 55–60 % removal of juice from the inherent liquid present in the fresh plant material (Arlabosse et al., 2011). Twin-screw extrusion has also been proposed for efficient mechanical pressing of alfalfa with more than 50 % of protein recovery in the pressed juice (Colas et al., 2013a, 2013b).

The degree of cell disruption is critical for the juice expression and protein recovery. However, about at least half of the proteins are retained in the press cake after the mechanical pressing. In this regard, several authors recommended the addition of water to the press cake and a secondary re-pressing in order to extract part of the retained proteins (Byers and Sturrock, 1965; Knuckles et al., 1972; Morrison and Pirie, 1961). Morrison and Pirie (1961) reported that half of the proteins left in the press cake could be extracted by means of a secondary pulping and pressing with addition of water. In the PRO-XAN process, the addition of water to the press cake and secondary re-pressing resulted in an increased protein recovery in the green juice from 40 % to 53 % (Knuckles et al., 1972). Re-extraction of alfalfa press cake was also performed with addition of dilute alfalfa solubles (5–6 %DM) which resulted in a press cake with reduced protein content (about 17 % reduction) and improved the total LPC yield (Edwards et al., 1977). According to Edwards et al. (1978), the effect of free water addition is greater than when the water is present in the plant cells. More recently, (Colas et al., 2013a) achieved the largest protein extraction from chopped alfalfa at the highest liquid-to-solid ratios in a twin-screw extruder in which water was added at different rates. Therefore, the addition of water likely favors the recovery of proteins in the green juice but at the same time, the concentration of proteins in the green juice is diluted. In addition, chopping the fresh plant material before the mechanical pressing has been recommended since it helps releasing plant soluble components (King et al., 2012).

In contrast, Morrison and Pirie (1961) recommended to carry out the pulping and pressing at alkali pH of around 8.0 for an improved extraction of proteins. Indeed, leaf protein extractability was highly influenced by pH according to Betschart and Kinsella (1973), who concluded that leaf protein is more soluble and chloroplasts are disrupted more effectively at high pH values; however, they suggested to use pH values between 7.0–8.0 to avoid the risk of protein denaturation. In the PRO-XAN process, freshly chopped alfalfa was treated with gaseous ammonia to increase the pH to 8.5 (Lazar et al., 1971). Addition of alkali could increase the protein extraction yields compared to water extraction and the efficacy of alkali conditions relies on the breakdown of the cell walls or on the breakdown of the protein itself (Sari et al., 2015a, 2015b). The temperature of the plant material before the mechanical pressing is also an important factor for the recovery of proteins during mechanical pressing, as studied by Hanna and Ogden (1980). Results

showed that heating to 35°C, 50°C or 60°C before mechanical pressing was detrimental for the juice expression and protein recovery while cooling to 3°C, 7°C or 14°C had no effect compared with the ambient temperature (25°C).

Clearly, the equipment design and operation conditions during the mechanical pressing of fresh plant material are crucial for ensuring an efficient juice expression and protein extraction into the green juice. The various extraction methods may also facilitate the co-extraction of anti-nutrients, which can influence animal performance.

3.2. Protein precipitation

The aim of the protein precipitation step is to concentrate the proteins into a solid fraction, which can be further separated and dried into a storable product (Bals et al., 2012). The precipitation of the proteins is usually performed by thermal coagulation or acidification but other methods including addition of flocculants or bacterial fermentation have been investigated and are detailed below.

The green juice contains chloroplast and cytoplasmic proteins from the plant cells (Fiorentini and Galoppini, 1983). The chloroplast proteins, also known as green proteins are the insoluble lipoproteins mostly present in the thylakoid membranes. These proteins are easy to destabilize and coagulate more rapidly at lower temperatures resulting in a dark green concentrate with a strong grassy flavor (Edwards et al., 1975; Fiorentini and Galoppini, 1983; Hernandez et al., 1988). The cytoplasmic white proteins are soluble in the cell cytoplasm or in the stroma and are relatively stable. Precipitation of the white protein fraction results in a tasteless, odorless white/creamy precipitate (Fiorentini and Galoppini, 1983). Two different strategies can be used for the precipitation of proteins: (a) an unfractionated LPC containing both green and white proteins can be obtained; (b) alternatively, a fractional process can be performed in order to firstly precipitate the green protein fraction into a LPC suitable for animal feeding and then, the white protein fraction into a LPC suited for human food (Chiesa and Gnansounou, 2011).

3.2.1. Heat coagulation

Heat coagulation has been widely applied for the precipitation of proteins from plant green juices at temperatures ranging from 60°C to 95°C (Baraniak, 1990; Byers and Sturrock, 1965; Collins, 1986; Edwards et al., 1975; Koschuh et al., 2004; Lazar et al., 1971; Morrison and Pirie, 1961). Heating provokes the coagulation of proteins as result of opening up hydrophobic sites and protein denaturation (Bals et al., 2012). According to Morrison and Pirie (1961), heat coagulation at 75 – 80°C by direct steam injection in the green juice was the most convenient method for producing leaf protein concentrates on a large scale and indeed, it was the method utilized in the Rothamsted process (Pirie, 1971). In the PRO-XAN process, the green juice produced from freshly chopped alfalfa was heat coagulated with steam to around 90°C for 2 – 3.5 min in order to produce a “coagulum” rich in proteins (49.0–53.2 % DM) and xanthophyll intended for non-ruminants feeding (Lazar et al., 1971; Spencer et al., 1971). Heat coagulation was also utilized for the fractional precipitation of the green and white protein fractions. In the PRO-XAN II process, the green juice was initially heated at 60°C for 20 s to agglomerate the green protein fraction (PRO-XAN II); then, the supernatant was heat coagulated with steam at 80°C into a white fraction concentrate (WELPRO) (de Fremery et al., 1973; Edwards et al., 1975). The protein content in the green protein concentrate and in the white protein concentrate was 47.2 %DM and 88.7 %DM, respectively. Two-step heat coagulation was also used for the precipitation of the green protein fraction, after heating at 60°C for 20 – 30 s, and the white protein fraction, after heating at 80°C 20 – 30 s, from green juices produced from different forages (Damborg et al., 2020). The resulting green and white protein concentrates presented similar protein contents between 24.5–40.4 %DM and 22.8–45.1 %DM, respectively.

Heat coagulation is an efficient method for the precipitation of proteins. Nevertheless, the leaf protein concentrates produced by heat coagulation might have low protein solubility due to the irreversible changes in the protein structure caused by denaturation, which could also affect other functional properties (Betschart and Kinsella, 1973; Bray and Humphries, 1978; Lamsal et al., 2007). The denaturation of Rubisco takes place at 76.2°C (Lamsal et al., 2007).

3.2.2. Acid precipitation

The addition of acid changes the solubility of the proteins in the green juice and can lead to their precipitation. Several authors have studied the solubility of leaf proteins under different pH values. For instance, Betschart and Kinsella (1973) studied the solubility curves for the total N and the protein N (i.e. TCA-insoluble N) from soybean leaves and suggested that acid precipitation might be a useful method for precipitation of proteins since less than 5 % of the protein N was soluble at the isoelectric point (pH 3.7). The isoelectric point refers to the pH value at which proteins have no overall charge (equal positive and negative charges) and thus minimum solubility; the isoelectric point of most proteins is between pH 4.5–6.5 (Coldebella et al., 2013). In cassava leaves, the minimum protein solubility and highest protein precipitation was achieved for pH values between 4.0–5.0 (Coldebella et al., 2013). The isoelectric point of Rubisco (i.e. fraction I protein) extracted and purified from tobacco, spinach, cotton and maize leaves was between pH 4.4–4.7 (Bahr et al., 1977). Furthermore, Merodio et al. (1983) investigated the effect of pH on the solubility of proteins and chlorophyll from spinach leaves. The minimum protein solubility was achieved at pH 4.0 with around 75 % of proteins precipitated while the minimum chlorophyll solubility was achieved between pH 3.7–4.0.

Acid precipitation has been performed in order to obtain an unfractionated LPC (Baraniak, 1990; Coldebella et al., 2013; Damborg et al., 2020; Morrison and Pirie, 1961); but it has also been combined with other precipitation methods to obtain green and white protein concentrates separately (Lamsal et al., 2007; Miller et al., 1975). Miller et al. (1975) utilized flash heating of the alfalfa green juice at 60°C for 20 s to remove the green chloroplast proteins followed by acid precipitation with HCl of the soluble white proteins. The protein yield in the protein concentrate (i.e. amount of proteins extracted in the protein concentrate relative to the amount of protein present in the green juice) was increased from 63 % to 87 % as pH in the green juice was decreased from 4.5 to 3.5.

Interestingly, the protein content in the protein concentrate (i.e. protein concentration in g protein/g dry matter) was decreased as well, indicating a greater precipitation of acid insoluble contaminants at lower pH values. Damborg et al. (2020) studied the precipitation of proteins under a pH range from 3.0–5.0 by addition of HCl to different green juices. In most cases, the precipitation efficiency (i.e. protein yield in the protein concentrate) was not pH-dependent except for red clover green juice, with the best precipitation efficiency achieved for pH 4.0. Furthermore, acidification showed a tendency towards a better precipitation efficiency when compared with heat coagulation (Damborg et al., 2020). A combination of heat coagulation at 55°C with acid precipitation with HCl to pH 3.5 to obtain green and white protein concentrates was performed in alfalfa green juice by Lamsal et al. (2007). Acidification with HCl (pH 3.3) was combined with heat treatment (100°C or 140°C) and/or with chemical treatment (zinc chloride) to reduce protein degradation in the production of LPC from orchard grass and switch grass (Kammes et al., 2011). The LPC contained less than 40 % protein on a DM basis (Kammes et al., 2011).

3.2.3. Addition of flocculants

The action of flocculants derives from their ability to aggregate particles, proteins in this case, forming large complexes that easily settle and can be partitioned from the mixture. The addition of flocculants to the green juice has been proposed to precipitate the proteins and produce LPC at room temperature avoiding heat coagulation (Anelli et al., 1977; Baraniak, 1990; la Cour et al., 2019). In the poly-protein process, a cationic polyelectrolyte was added to alfalfa green juice, previously acidified to pH 4.5 with HCl, provoking the instant flocculation and coagulation of proteins, later separated by filtration (Anelli et al., 1977). The process yielded 7.5 kg of wet protein concentrate from 100 kg of fresh plant and the LPC contained a crude protein content of 56.2 %DM (Anelli et al., 1977). Baraniak (1990) also studied the utilization of anionic and cationic flocculants for the precipitation of proteins from alfalfa green juice at room temperature in an effort to develop a more economical process compared to heat coagulation and acid precipitation. The protein content in the LPC produced from alfalfa varied slightly with the different protein precipitation methods i.e. 42.7 %DM with cationic flocculants, 42.9 %DM with acidification (pH 3.5), 45.0 %DM with anionic flocculants and 53 %DM with heat coagulation (85°C). Several flocculants were tested in green juices from alfalfa, tall fescue and ryegrass for the destabilization of the chloroplast protein fraction before obtaining a white protein concentrate by acid precipitation with HCl to pH 4.0 (Bray and Humphries, 1979). Besides, the use of cationic flocculants resulted in an improved separation of the chloroplast protein fraction from alfalfa green juice without the need for heating (Knuckles et al., 1980). A recent work studied the potential use of lignosulfonates, a by-product of the sulfite pulping process, as a flocculant for the aggregation and precipitation of proteins (la Cour et al., 2019). The optimal lignosulfonate dose for an efficient protein precipitation was 0.6–0.7 g lignosulfonate per g protein, which resulted in LPC with protein contents of 25.7 %DM for ryegrass and 39.0 %DM for red clover. The lignosulfonate content in the LPC was relatively high and accounted for around 25 %DM (la Cour et al., 2019).

3.2.4. Bacterial fermentation

The production of organic acids in green juice itself by bacterial fermentation is an interesting alternative to the addition of acids for decreasing pH and precipitating the proteins. During fermentation of the green juice, an initial lag phase with constant pH was observed followed by a continuous pH drop phase, until inhibition took place (Ajibola, 1984). A clear correlation between pH and lactic acid concentration in the fermented juice indicated that lactic acid fermentation was responsible for the pH drop. A deep-green precipitate was observed in the fermented juice when the pH was around 4.5 and, further pH to around 3.6 resulted in a thin layer of light yellow precipitate (Ajibola, 1984). Consequently, lactic acid fermentation of green juice may be utilized for the precipitation of proteins. In addition, Ajibola (1984) compared natural fermentation of green juice with inoculation of lactic acid bacteria strains (i.e. *Lactobacillus plantarum* and *Pediococcus cereviseae*). Results showed that lactic acid bacteria inoculation was significant for reducing the lag phase and for achieving a lower pH value at the end of the fermentation.

Several *Lactobacillus* strains were screened for continuous acidification of brown juice (i.e. unsterile plant juice pressed in a crop-drying factory) (Thomsen and Kiel, 2008). *Lactobacillus salivarius* BC 1001, previously isolated from grass juice, resulted as the most promising strain for a fast pH decrease due to its short lag phase and high lactic acid production rate compared with other *Lactobacillus* strains (Thomsen and Kiel, 2008). Later on, Kiel et al. (2015) patented a process utilizing *L. salivarius* BC 1001 for lactic acid fermentation of green juice in order to lower the pH and precipitate proteins into a LPC containing functional non-denatured proteins. Lactic acid fermentation of green juice with inoculated culture of *L. salivarius* was compared with natural fermentation and acidification with H_2SO_4 for the precipitation of proteins (Santamaría-Fernández, 2015). Similar protein recovery yields were obtained in the protein concentrates for the different precipitation methods. Green juices produced from red clover, clover grass, alfalfa and oilseed radish were fermented with *L. salivarius* to final pH between 4.0–4.7 resulting in LPC with protein contents ranging from 39 to 46 %DM (Santamaría-Fernández et al., 2017). Lactic acid fermentation of the green juice requires less energy than heat coagulation and does not involve harsh conditions, which may damage the proteins.

3.2.5. Other protein precipitation methods

Direct spray-drying of green juice was proposed as an alternative method for producing LPC while preserving valuable soluble components and avoiding drying at high temperatures (Hartman et al., 1967). Relatively high N recoveries from the total plant N (43–44 %) were achieved in the freeze-dried product, which contained between 18–35 % protein and high concentration of vitamins. Furthermore, chlorophyll was removed from the spray-dried product with 95 % ethanol resulting in an increased protein content in the freeze-dried product (26–43 %). Nevertheless, direct freeze-drying of the green juice was refused by Pirie (1971), due to the risk for the formation of indigestible complexes and the presence of harmful soluble compounds.

Alternatively, ultrafiltration of the green juice with ceramic membranes was studied as an alternative to heat coagulation in order

to produce a protein concentrate with higher solubility (Kromus et al., 2004). The protein content was between 26.3–38.8 %DM in the LPC produced by ultrafiltration and between 40.1–46.3 %DM in the LPC produced by heat coagulation. However, protein degradation played a significant role during ultrafiltration, which should be carried out at low temperatures or in short batch cycles to avoid protein degradation. Ultrafiltration of a clarified alfalfa juice, obtained after removing the heat coagulated green protein fraction, was carried out with a 10 kDa cut-off membrane for separating the white protein concentrate (Lamsal et al., 2007).

Freezing alfalfa green juice at -25°C, followed by centrifugation of the thawed juice, allowed the production of a freezing curd, which contained 50 %DM and 60 % of N from the juice (Hernandez et al., 1995). The freezing curd was treated with 2-propanol to increase the protein content and produce a chlorophyll-free concentrate (Hernandez et al., 1995). Organic solvents were also utilized in order to flocculate the chloroplast green proteins at room temperature and later, precipitate the white soluble proteins with HCl to pH 4.0 at 40°C (Bray and Humphries, 1978). The protein content in the resulting LPC was about 55 % in the green concentrate and about 60 % in the white concentrate. Nevertheless, the yield of white protein concentrate was only one part of white protein to three parts of green protein in alfalfa, or to seven parts of green protein in fescue and ryegrass.

3.3. Protein concentration: separation of proteins and drying

In most cases, the separation of proteins from the juice is achieved by centrifugation but filtration processes or membrane technology can be utilized as well. Afterwards, the LPC is usually dried in order to produce a stable product that can be stored and easily transported. In case that drying or freezing are not performed, growth of soil fungi like *Mucus racemosus*, which are not inhibited during preparation of the LPC, may cause microbial spoilage of the wet protein concentrate (Arkcoll, 1973).

Drying significantly influences the texture and the nutritional quality of the LPC (Morrison and Pirie, 1961). Indeed, a decreased nutritional value in the LPC has been observed upon drying at high temperatures (Miller et al., 1972; Morrison and Pirie, 1961). Heat treatments of LPC can lead to Maillard reactions between reducing sugars and lysine, rendering lysine biologically unavailable and reducing the overall nutritional value (Gilani et al., 2012). For instance, cooking of different legume seeds for 2 h in the presence of glucose resulted in losses of available lysine of up to 38 % (Almas and Bender, 1980). Moreover, heat (or alkali) treatments of food proteins can provoke racemization of amino acids to D-enantiomers and concurrent formation of lysinoalanine (LAL, an unnatural amino acid derivative) (Gilani et al., 2012; Schwass and Finley, 1984). Schwass and Finley (1984) studied the racemization and LAL formation in alfalfa LPC under a range of temperatures, pH and times and concluded that serine and aspartate were the most susceptible amino acids for racemization at lower temperatures, that racemization is more likely in denatured proteins and that the formation of LAL mostly takes place under alkali conditions.

Miller et al. (1972) compared different methods for drying alfalfa LPC, including freeze-drying, spray-drying and different air-drying devices. Freeze-drying resulted in the least deterioration of nutrients and the highest concentration of carotenes and xanthophyll in the LPC although it was expensive to run at large scale. Very fast freezing of LPC was achieved by spreading the LPC in very thin layer and was recommended by Morrison and Pirie (1961) for an improved texture compared to normal freeze-drying. Drum drying resulted in scorching and burning of the LPC (Miller et al., 1972), while drying in a current of low temperature yielded a hard and granular LPC (Morrison and Pirie, 1961). Spray drying resulted in a dried LPC with a similar texture to the freeze-dried LPC and just a minor loss of nutrients compared to freeze-drying (Miller et al., 1972). Heat treatment of LPC at 121°C for 45 min decreased the total content of amino acids, and specifically affected certain amino acids such as arginine, histidine, lysine and methionine (Chowdhury et al., 2018). In some cases, heat treatment also reduced the solubility of proteins in LPC and Chowdhury et al. (2018) concluded that LPC are extremely heat labile.

Wet preservation of LPC was investigated by addition of acetic acid, lactic acid and sodium chloride to avoid microbial growth (Arkcoll, 1973). Acetic acid resulted the most efficient preservative to avoid microbial growth while significant higher concentrations of lactic acid or salt were required to inhibit the growth of fungi. Dry preservation of LPC was studied to reduce the oxidative spoilage, which led to losses of beta-carontene and unavailability of certain amino acids due to co-oxidation with lipids (Arkcoll, 1973). In this regard, cold storage and oxygen exclusion by vacuum packing resulted in considerably lower beta-carontene losses than open storage at room temperature.

The drying and preservation of the LPC is crucial for maintaining the nutritional value and stability and therefore an adequate method should be utilized.

3.4. Protein extraction yields

A summary of protein extraction yields achieved from different plant biomasses into LPC produced by different extraction procedures is presented in Table 1.

The protein extraction yield from the fresh plant material into the LPC is highly influenced by the extraction procedure. Mostly, an efficient mechanical pressing ensures a great release of proteins into the juice; however, the subsequent protein precipitation and protein concentration are also important to ensure an overall high protein extraction yield. Apart from the protein extraction procedure, the yield of extractable proteins is also highly influenced by agronomic factors such as the plant species, variety, type of soil and its fertility, growth stage and age of the plant at harvesting, climate or plant density (Arkcoll and Festenstein, 1971). For instance, legume species (white clover, red clover or alfalfa) showed higher crude protein contents than grass species (ryegrass or tall fescue); but for the five plant species, the potentially extractable protein decreased with maturity across spring growth (Solati et al., 2017). Furthermore, plant leaves presented higher content of crude protein and extractable protein than plant stems (Solati et al., 2018). A recent study investigating the extraction of proteins from three taxonomically distant plant species (chicory, red clover and timothy)

Table 1
Summary of protein recovery yields achieved by different protein extraction procedures.

Plant biomass	Protein extraction method	Protein recovery yield [a] %, from GJ into LPC	Protein recovery yield [b] %, from plant into LPC	Comments	References
Alfalfa	Pulping and pressing + direct spray-drying		43.0 %		Hartman et al., 1967
Alfalfa	Twin-screw pressing + fractional heating (60°C for 20 s; 80°C)		23.8 % (15.1 % green proteins + 8.7% white proteins)	PRO-XAN II process in pilot plant	Edwards et al., 1975
Alfalfa	Twin-screw pressing + heat coagulation (60°C) + acidification (pH 4.5 − 3.5)	63 – 87 %		Yield for the acidification step after removal of green protein fraction	Miller et al., 1975
Alfalfa	Screw pressing + acidification with HCl (pH 4.5) + cationic polyelectrolyte		20 %	Yield estimated from concentrate yield and fractions composition	Anelli et al., 1977
Alfalfa	Screw pressing + heat coagulation (95°C)	~ 50 %		Approximate values taken from a graph	Koschuh et al., 2004
Alfalfa	Screw pressing + ultrafiltration	~ 50 %		Approximate values taken from a graph	Koschuh et al., 2004
Alfalfa	Screw pressing + lactic acid fermentation (*L. salivarius*)	38.6 %	15.1 %		Santamaría-Fernández et al., 2017
Alfalfa	Twin-screw pressing + acidification with HCl (pH 4.0)	63.5 %			Damborg et al., 2020
Alfalfa	Twin-screw pressing + fractional heating (60°C for 20 − 30 s; 80°C for 20 − 30 s)	61.0 % (58 % green proteins + 3% white proteins)			Damborg et al., 2020
Chicory	Twin-screw pressing + lactic acid fermentation (*L. salivarius*)	79 − 83%	36 − 40%	Yields for different development stages of plants at harvest time	Santamaría-Fernández et al., 2019b
Clover grass	Screw pressing + lactic acid fermentation (*L. salivarius*)	51.7 %	17.1 %		Santamaría-Fernández et al., 2017
Clover grass	Screw pressing + lactic acid fermentation (*L. salivarius*)	29.5 %	6.9 %	Demonstration-scale single case production	Santamaría-Fernandez et al., 2019a
Oilseed radish	Screw pressing + lactic acid fermentation (*L. salivarius*)	43.7 %	12.1 %		Santamaría-Fernández et al., 2017
Orchadgrass	Screw pressing + acidification with HCl (pH 3.3)	~ 30 %		Approximate values taken from a graph	Kammes et al., 2011
Pea vines	Pulping and pressing + direct spray-drying		43.9 %		Hartman et al., 1967
Red clover	Pulping and pressing + heat coagulation (80°C)		44.4 %*	Rothamsted process. *Average values (n = 5)	Byers and Sturrock, 1965
Red clover	Screw pressing + lactic acid fermentation (*L. salivarius*)	66.7 %	23.4 %		Santamaría-Fernández et al., 2017
Red clover	Twin-screw pressing + lignosulfonate (~ 4 N eq) + acidification with HCl (pH 2 − 3)	78 %			la Cour et al., 2019
Red clover	Twin-screw pressing + heat coagulation (80°C for 30 s)	76 %			la Cour et al., 2019
Red clover	Twin-screw pressing + acidification with HCl (pH 4 − 4.5)	77 %			la Cour et al., 2019
Red clover	Twin-screw pressing + lactic acid fermentation (*L. salivarius*)	72 − 80%	40 − 42%	Yields for different development stages of plants at harvest time	Santamaría-Fernández et al., 2019b

(*continued on next page*)

Table 1 (*continued*)

Plant biomass	Protein extraction method	Protein recovery yield [a] %, from GJ into LPC	Protein recovery yield [b] %, from plant into LPC	Comments	References
Red clover	Twin-screw pressing + acidification with HCl (pH 4.0)	71.9 %			Damborg et al., 2020
Red clover	Twin-screw pressing + fractional heating (60°C for 20 − 30 s; 80°C for 20 − 30 s)	63.3 % (60 % green proteins + 3% white proteins)			Damborg et al., 2020
Ryegrass	Screw pressing + heat coagulation (95°C)	~ 45 %		Approximate values taken from a graph	Koschuh et al., 2004
Ryegrass	Screw pressing + ultrafiltration	~ 60 %		Approximate values taken from a graph	Koschuh et al., 2004
Ryegrass	Twin-screw pressing + lignosulfonate (~ 4 N eq) + acidification with HCl (pH 2 − 3)	73 %			la Cour et al., 2019
Ryegrass	Twin-screw pressing + heat coagulation (80°C for 30 s)	68 %			la Cour et al., 2019
Ryegrass	Twin-screw pressing + acidification with HCl (pH 4 − 4.5)	70 %			la Cour et al., 2019
Ryegrass	Twin-screw pressing + acidification with HCl (pH 4.0)	60.4 %			Damborg et al., 2020
Ryegrass	Twin-screw pressing + fractional heating (60°C for 20 − 30 s; 80°C for 20 − 30 s)	58.9 % (55 % green proteins + 4% white proteins)			Damborg et al., 2020
Switchgrass	Screw pressing + acidification with HCl (pH 3.3)	~ 25 %		Approximate values taken from a graph	Kammes et al., 2011
Timothy	Twin-screw pressing + lactic acid fermentation (*L. salivarius*)	76 − 86%	24 − 26%	Yields for different development stages of plants at harvest time	Santamaría-Fernández et al., 2019b
White clover	Pulping and pressing + heat coagulation (80°C)		39.3 %*	Rothamsted process. *Average values (n = 12)	Byers and Sturrock, 1965
White clover	Twin-screw pressing + acidification with HCl (pH 4.0)	71.8 %			Damborg et al., 2020
White clover	Twin-screw pressing + fractional heating (60°C for 20 − 30 s; 80°C for 20 − 30 s)	61.0 % (59 % green proteins + 2 % white proteins)			Damborg et al., 2020

[a] Percentage of total proteins in the green juice (GJ) that are recovered in the LPC.
[b] Percentage of total proteins in the fresh plant that are recovered in the LPC.

found that the plant species and development stage significantly influenced the protein extractability (i.e. amount of CP extracted per kg DM plant) and production of LPC (i.e. amount of dry LPC produced per kg DM plant) (Santamaría-Fernández et al., 2019b). Best results were obtained for red clover given its high protein content and relatively low DM content. Protein extractability and production of LPC were reduced with increased plant maturity because of the accumulation of DM and reduction of crude protein (Santamaría-Fernández et al., 2019b, 2019a). According to Edwards et al. (1977), the LPC yield was affected by the amount of juice extracted during mechanical pressing as well as by other factors such as the biomass temperature, fiber content and dry matter content. The highest yields were achieved for cool weather, low fiber content and high moisture. The temperature effect may influence the extent of proteolysis, which may be minimized by cool temperatures and short waiting time between harvesting and further processing (Edwards et al., 1977).

4. The LPC: nutritional value for monogastric animals

The protein concentration of the resulting LPC can vary significantly depending on the extraction procedure and the plant biomass. Protein contents ranging from 18 %DM to 89 %DM were obtained with the different protein precipitation methods detailed in Section 3.2. LPC are also good sources of lipids, beta-carotene and xanthophyll. In addition, “unfractionated” LPC also contains cell debris such as fibers, broken chloroplasts, and particulates (Tamayo Tenorio et al., 2018).

The feeding value of the leaf protein concentrate depends on the protein concentration and quality but also on the energy content and on the presence of anti-nutritional compounds and their concentration (Dale et al., 2009). As mentioned earlier, beta-carotene and xanthophyll can be beneficial for e.g. yolk color. Depending on the age and type of animal, the presence of fibers can be unwanted; i.e. young animals such as new hatched chickens, pigs and calves have very high nutritional requirements regarding digestibility during weaning and are very sensitive to anti-nutritional compounds, including fiber.

4.1. Amino acid composition

Proteins are sources of amino acids, which can be divided into essential, semi-essential, and non-essential amino acids. Essential amino acids are those that cannot be synthetized by the organism itself, but need to be supplied with the diet in adequate amounts and ratio to each other for building up the body’s own proteins (Sundrum et al., 2005). In pig nutrition, nine out of the total twenty different amino acids found in nature are essential for the animals maintenance and growth i.e. histidine (HIS), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), phenylalanine (PHE), threonine (THR), tryptophan (TRP) and valine (VAL) (Boisen, 1997). The same nine amino acids together with arginine (ARG) are essential in poultry feeding (Blair, 2008). Furthermore, cysteine (CYS) and tyrosine (TYR) are semi-essential amino acids for pigs and poultry since they can only be synthesized from methionine and phenylalanine, respectively (Sundrum et al., 2005). The deficit of any essential amino acid in the animal diets results in a general protein deficiency (Blair, 2008) and therefore, the adequate supply of essential amino acids is crucial in the nutrition of monogastric animals.

The profile of essential amino acids (except for tryptophan) in LPC extracted from alfalfa or red clover by means of different precipitation methods is shown in Fig. 6 and compared with the profile of essential amino acids in Rubisco (Baraniak, 1990; Chowdhury et al., 2018; Collins, 1986; Hartman et al., 1967; Prevot-D’Alvise et al., 2004; Santamaría-Fernández et al., 2017; Stødkilde et al., 2019). Overall, essential amino acids represent about 57 % of the total sum of amino acids in Rubisco while essential amino acids ranged between 46–53 % of the total sum of amino acids in the different LPC. It is likely that Rubisco is the main protein in protein concentrates extracted from plant biomass but the minor differences observed in the profile of essential amino acids reveal that other proteins are extracted as well. The relatively high proportion of essential amino acids in the protein concentrates indicates a suitable supply of essential amino acids for monogastric animals, and is comparable with the percentage of essential amino acids in soybeans (47–48 %) (Steenfeldt and Hammershøj, 2015). LPC produced by heat coagulation (80°C) from alfalfa and red clover presented a very similar proportion of amino acids (Collins, 1986). In the same way, LPC produced by lactic acid fermentation from alfalfa and red clover also showed a similar proportion of amino acids (Santamaría-Fernández et al., 2017). Indeed, a larger uniformity in the amino acid composition was observed between LPC than in the whole crops (Collins, 1986). The proportion of essential amino acids is similar between LPC extracted by different precipitation methods with some exceptions. For instance, LPC produced from alfalfa by heat coagulation (85°C) (Baraniak, 1990), presented the lowest proportion of lysine and the highest proportion of phenylalanine compared with LPC produced by other protein precipitation methods. Overall, the LPC from different plants present a similar profile of essential amino acids despite the plant biomass or the protein precipitation method, which is advantageous for commercializing the product for animal feeding purposes.

Monogastric animals have specific requirements for each essential amino acid. Lysine is the first limiting amino acid in most pig diets, which mainly consist of cereals having low levels of lysine (Studnitz, 2019). Methionine is commonly the first limiting amino acid in poultry given the high methionine requirements for feather production (Studnitz, 2019). For instance, most protein sources for feeding organic laying hens are deficient in methionine and lysine (van de Weerd et al., 2009). In particular, low-methionine diets in the feeding of laying hens can result in a reduced mass egg output or a detrimental effect on immune-competence and feather pecking (van de Weerd et al., 2009). However, it is difficult to supply enough methionine without increasing the protein content excessively, which results in an increased N excretion (Studnitz, 2019). The level of the first limiting amino acid determines the utilization of the other essential amino acids (Blair, 2008) and increasing the supply of one amino acid improves the animal performance only if no

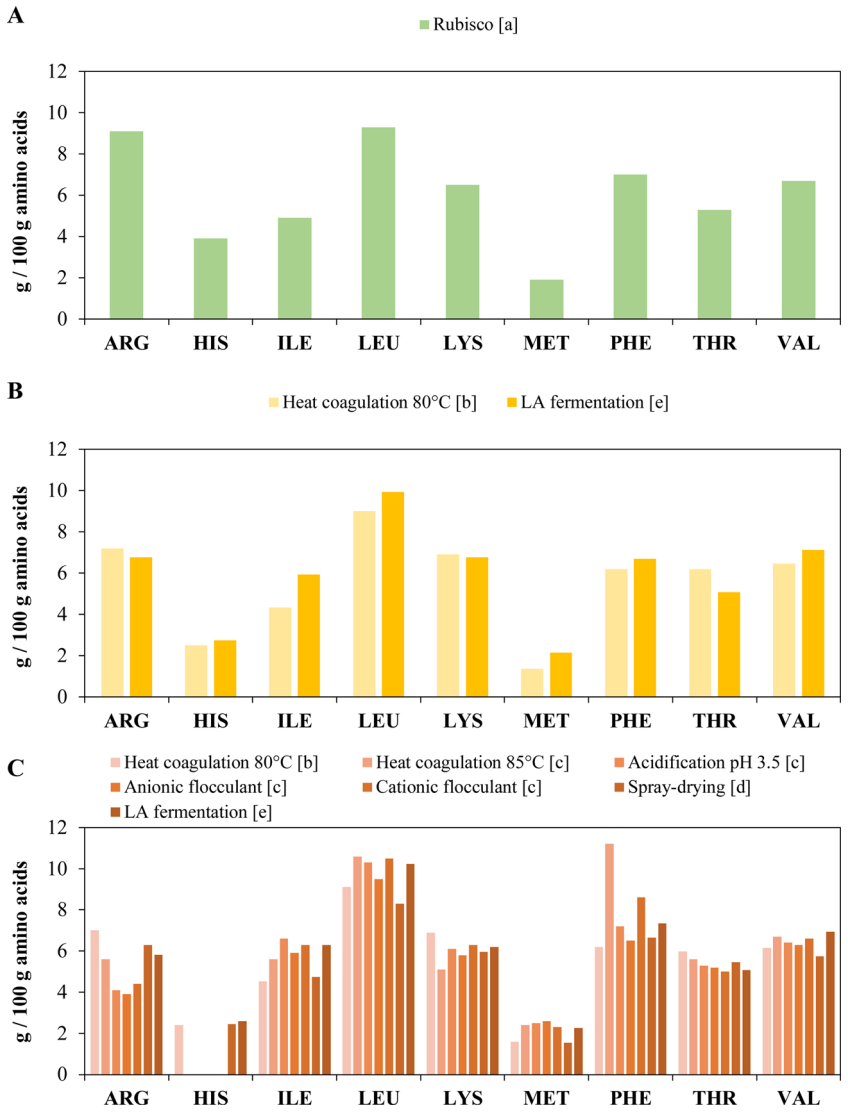

Fig. 6. Proportion of essential amino acids in Rubisco (A) and in leaf protein concentrates produced by different precipitation methods from red clover (B) and alfalfa (C) according to the literature.
[a] Prevot-D'Alvise et al., 2004; [b] Collins, 1986; [c] Baraniak, 1990; [d] Hartman et al., 1967; [e] Santamaría-Fernández et al., 2017.

other amino acid is limiting (Schutte and De Jong, 1999). Therefore, it is important to consider the requirements for all essential amino acids in the formulation of the animal diet, especially in organic production where the use of synthetic amino acids is not allowed.

The concept of ideal protein, widely used in pig and poultry feeding, refers to the condition for all essential amino acids being co-limiting so that the amino acid supply matches the amino acid requirement (van Milgen and Dourmad, 2015). In the ideal protein, the requirement for each essential amino acids is expressed relative to the requirement for lysine assuming that the amino acid to lysine ratio is not affected by dietary, environmental and genetic factors (Schutte and De Jong, 1999; van Milgen and Dourmad, 2015). The ideal protein profiles for pigs and poultry found in the literature (Bedford, 2016) is presented in Table 2 together with the protein profile estimated for LPC and for some common protein sources currently available for feeding monogastric animals. The ratio for each amino acid is expressed relative to lysine of digestible basis (SID) for growing pigs or of true digestibility (TD) for poultry (Bedford, 2016). The ratio for each amino acid was estimated relative to the total lysine content for LPC, soybeans, soybean meal and grain legumes (faba bean, pea and lupin). Soybeans are the main protein source for feeding organic poultry (Hammershøj and Steenfeldt, 2005), while soybean meal is the most important protein source for feeding monogastric animals in conventional farming because of the high proportion of essential amino acids with high digestibility (Sundrum et al., 2005). Based on our estimations, LPC are relatively good sources of sulfur-amino acids (MET + CYS) compared to soybean meal, soybeans and lupine. In particular, LPC produced from alfalfa showed the best ratio of sulfur-amino acids i.e. equal or superior to soybean meal. However, the relative ratio of methionine + cysteine is still below the requirements for pigs and poultry. Furthermore, the LPC present good relative ratios of

Table 2
Ideal amino acids profile expressed as percentage of lysine.

	Ideal protein profile			Leaf protein concentrates				Soybeans[d]	Soybean meal[d]	Grain legumes		
	Growing pigs[1, a]	Broilers[a]	Laying hens[a]	Alfalfa[b]	Alfalfa[c]	Red clover[b]	Red clover[c]			Faba bean[e]	Pea[e]	Lupin[e]
LYS	100	100	100	100	100	100	100	100	100	100	100	100
MET + CYS	60	75	42	46	52	40	41	42	46	31	33	45
THR	65 – 68	65	60	75	82	76	75	60	64	57	53	73
TRP	19 – 22	17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
VAL	65 – 70	80	69	98	112	102	105	69	77	72	66	82
ILE	53	67	71	82	101	83	88	71	73	64	58	85
LEU	100	105	112	136	165	139	147	112	124	116	101	148
HIS	32	40	39	38	42	36	40	39	44	42	35	56
PHE + TYR	95	105	75	92[2]	119[2]	91[2]	99[2]	75[2]	82[2]	68[2]	68[2]	83[2]
ARG	42	105	120	94	93	92	100	120	120	143	121	224

[1] The recommended ratio increases with increasing weight of the growing and finishing pigs.
[2] Based on the phenylalanine content only.
[a] Bedford, 2016; [b] produced by acidification to pH 4.0 (Stødkilde et al., 2019); [c] produced by fermentation (Santamaría et al., 2017); [d] Steenfeldt and Hammershøj, 2015; [e] Jezierny et al., 2010.

threonine, histidine, phenylalanine + tyrosine, and arginine compared with the ideal amino acids profile for pigs and poultry, while the ratio for valine, isoleucine and leucine are above the requirements. In general, the profile for most amino acids was better than in soybean meal, soybeans and grain legumes, except for arginine with a lower relative ratio.

Therefore, LPC may be good sources for dietary protein, with relatively high content of methionine and cysteine, for feeding monogastric animals. Possible deficiencies in specific amino acids might be compensated from the diet by preparing mixed formulations with other protein sources such as lupin, which shows a relatively good amino acid profile compared with other grain legumes. Nevertheless, the amino acid content itself does not provide enough information about the nutritional value since the bioavailability of amino acids is required in relation with digestibility as well (Sundrum et al., 2005). Up to date, few works have studied the digestibility of specific amino acids in LPC so it is difficult to fully assess the nutritional value of LPC. A recent work evaluated the digestibility of dry matter and the standardized N digestibility of LPC produced from different biomasses (Stødkilde et al., 2019). The digestibility of dry matter ranged from 61 % to 76 % and the standardized N digestibility was between 75–85 %, with the highest digestibility values obtained for alfalfa LPC. Amino acid digestibility was determined in ileum-fistulated pigs using LPC produced from perennial ryegrass, red clover and alfalfa (Lærke et al., 2019). The ideal digestibility of essential amino acids was significantly lower than values for soybean meal because of a low protein content (33–38 %DM) in the LPC (Lærke et al., 2019).

To the authors' knowledge, LPC have already been tested in animal feeding trials with pigs and poultry. LPC produced from grass-clover was included in feed mixtures for organic broilers at different inclusion rates (Stødkilde et al., unpublished results). Results showed that 13 % of the crude protein in the feeding mixture could be substituted by the LPC; however, growth, feed intake and slaughter weight were reduced when increasing inclusion level to 26 % and to 39 %. The reduced animal performance was probably consequence of a low content of sulfur-amino acids and a high content of protein-bound in insoluble complexes unavailable to the broilers (Stødkilde et al., unpublished results). A similar LPC with inclusion levels of 0 (control), 4, 8 and 12 % in feed mixtures was added to the feed for layers at the expense of soybeans and soy cake. The 12 % clover grass protein feed contained no soybeans. The layers were fed with the four experimental mixtures through 12 weeks (hen age 30–41 weeks). Egg production showed no difference between the four treatments, and no difference in egg weight or feed utilization (kg feed/kg egg) was found. Throughout the trial period, the feathers and the pillows of the layers were very fine (Steenfeldt and Lübeck, 2018)

LPC with a higher protein content (47 %DM) was tested for feeding organic pigs in a study with 48 pigs (Stødkilde et al., unpublished results). Results showed that the LPC could substitute soybean press cake without any adverse effects on animal performance and meat quality.

5. Integration of leaf protein extraction with green biorefineries

The integration of leaf protein extraction within green biorefineries represents a great opportunity for the industrial development and establishment of protein extraction processes for feeding purposes, while facilitating the utilization of side stream fractions for production of chemicals and/or fuels and making the whole biorefinery process sustainable and economically profitable. Moreover, existing agricultural structures utilized in grassland cultivation such as the green crop drying plants will facilitate the implementation of green biorefineries (Kamm et al., 2016). The establishment of green biorefineries will also contribute to the preservation of cultural landscapes and biodiversity and will help in climate change mitigation and reduction of soybean imports (Höltinger et al., 2014; Kamm et al., 2016; Parajuli et al., 2015).

The idea behind the green biorefinery concept is to use fresh green biomass as feedstock for the integrated production of chemicals, materials, food and feed, energy and biofuels (Fig. 7) (Mandl, 2010). Due to the wet nature of the feedstock, green biorefineries involve an initial wet fractionation step in order to separate the green biomass compounds in its natural form (Kamm and

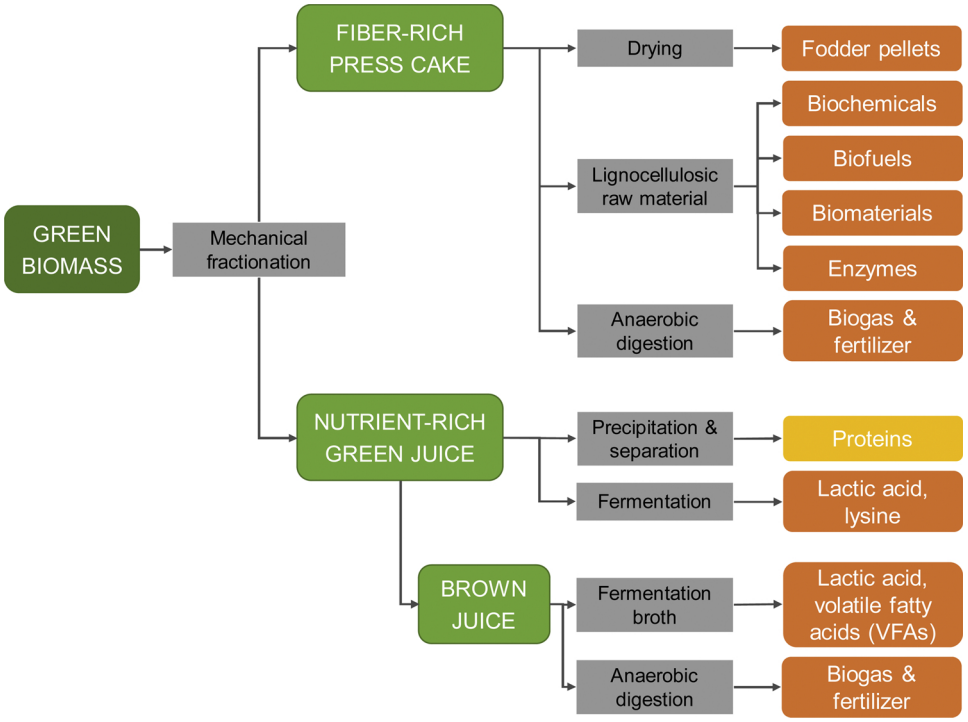

Fig. 7. Schematic diagram of green biorefineries for the production of chemicals, materials, food and feed, energy and biofuels from green biomass.

Kamm, 2004; Kromus et al., 2006). The wet fractionation or mechanical pressing results in the production of a fiber-rich press cake and a nutrient-rich green juice while minimizing the costs related with the biomass drying (Sharma et al., 2011). The press cake contains cellulose, starch, dyes, pigments, crude drugs and other organic compounds; besides, the green juice is rich in water soluble compounds such as proteins, free amino acids, organic acids, dyes, hormones, minerals, high quality crude drugs, and other organic substances (Kromus et al., 2006).

5.1. Availability of the green biomass

Green biomass includes grass from permanent grasslands, nature reserves and fallow land cultivation; forage crops like alfalfa or clover; and immature cereals (Kamm and Kamm, 2004; Kromus et al., 2006) as well as leaves from agro-industrial crops like sugar beet or cassava (Tamayo Tenorio et al., 2018). The relatively high content of aqueous cell juice containing abundant carbohydrates and proteins, and the relatively low content of lignin in the cell walls make green biomasses suitable feedstocks for green biorefinery purposes (Kromus et al., 2006).

In Europe, grassland is highly important for the livestock sector as feed for ruminant animals (i.e. beef, dairy and sheep), but it also shapes the landscape and positively contributes to the protection of biodiversity, nature, soil and water (Sharma et al., 2011). In 2016, around 60.5 million hectares of permanent grassland were grown in the EU representing one third of the total agricultural land area (EUROSTAT, 2020). Although the area under permanent grassland has been steady for several years in the EU, the share of organic permanent grassland is increasing considerably and around 5.3 million hectares of organic permanent grassland were cultivated in 2016 (EUROSTAT, 2020). Nevertheless, scarce improvements in the productivity of permanent and temporary grasslands have been achieved during the last decades in Europe compared with other agricultural crops (Smit et al., 2008). Furthermore, the establishment of the milk quotas provoked a decreased demand for grass in Europe, since grassland is important for milk production (Smit et al., 2008). In this context, new uses are needed for the surplus grass and for involving farmers in the grassland preservation and therefore, green biorefineries could represent an attractive alternative (Kromus et al., 2006; Mandl, 2010).

Forage legumes like alfalfa, clover and clover grasses are widespread crops utilized in crop rotations especially in organic farming due to their residual effect with N-supply and sanitation for the succeeding crops, and for feeding ruminants. The biological fixation of atmospheric N carried out by legumes, in symbiosis with rhizobia, provides soils and plants with N reducing the need for N inorganic fertilizers (Carlsson and Huss-Danell, 2003) representing a great advantage for the cultivation of legume crops. Nevertheless, the use of forage legumes has decreased significantly in Europe in the last decades (Doyle and Topp, 2004; Stoddard, 2013). The decline was driven by the availability and low cost of N inorganic fertilizers reducing the need for biological N fixation in crop rotations together with the fact that soybean import is a cheap protein source for livestock feeding (Stoddard, 2013). The utilization of legume crops in green biorefineries may encourage their cultivation favoring thus a more sustainable agriculture. A cost-benefit analysis showed that the production of fodder legumes in a crop rotation system is more profitable when the fodder legumes are

utilized as feedstock for a green biorefinery compared with market crop production with and without fodder production (Papendiek et al., 2016). Alfalfa is the most important forage crop in Europe and its high protein content and favorable amino acid composition makes it an excellent candidate for LPC production (Kamm et al., 2016). A recent study evaluated the environmental impact for the utilization of different green biomasses (grass-clover, ryegrass, alfalfa and festulolium) in a green biorefinery plant producing feed and energy (Corona et al., 2018). Based on Life Cycle Assessment (LCA), alfalfa showed the best overall performance in terms of environmental impact given its lower fertilization rate and higher yields compared with the other green biomasses evaluated.

Nevertheless, the fact that the green biorefinery aims at utilizing wet green biomass right after harvesting restricts it to seasonal operation, representing one of the main challenges to be faced. Alternatively, the utilization of grass silage as feedstock has been proposed, especially for winter operation of the green biorefinery plants (Ecker et al., 2012; Kamm et al., 2016). The utilization of grass silage yields different end products because of the cell wall degradation and lactic acid fermentation during the ensiling process (Kromus et al., 2006), and a lower protein yield is expected from silage juice compared to grass juice (Sharma et al., 2011).

5.2. Potential products

A broad range of products can potentially be produced in green biorefineries (Fig. 7). The press cake can be used for the production of pulp silage (Damborg et al., 2018) and fodder pellets for ruminants feeding, as raw material for production of chemicals or as feedstock for biogas production and later generation of heat and electricity (Kamm and Kamm, 2004). The green juice can be utilized for the extraction of high quality proteins with application in animal feeding but also, in the food and cosmetics industries (O'Keeffe et al., 2011; Parajuli et al., 2015). The green juice has proven to be suitable source of nutrients for fermentation to produce lysine as fodder additive (Thomsen et al., 2004), as well as to produce lactic acid that can be further utilized for manufacturing polylactic acid (PLA), which is a renewable and biodegradable plastic (Dietz et al., 2016; Papendiek and Venus, 2014; Vodnar et al., 2010). Alternatively, the "brown juice" i.e. the juice obtained after the extraction of proteins or after the lactic acid fermentation can be further utilized for the production of volatile fatty acids (Weimer and Digman, 2013) or as substrate for the production of biogas (Santamaría-Fernández et al., 2018). The utilization of the residual fractions in a biogas plant allows the production of energy in the form of methane while the digestate can be applied in the fields as fertilizer allowing the recycling of plant nutrients.

This multi-product approach in the green biorefineries contributes to create sufficient revenue so as to run a green biorefinery plant, compared to the production of protein concentrates alone (Mandl, 2010).

5.3. Green biorefineries in Europe

In Europe, there is a growing interest on the development of a bioeconomy and on the establishment of biorefineries to produce energy, chemicals, materials, food and feed products from any kind of renewable feedstock as alternative to fossil fuels and oil refineries. For instance, the European Commission launched The Bioeconomy Strategy and its Action plan in 2012 for the establishment of a bioeconomy that supports the production of renewable biological resources and their conversion into value added products (European Commission, 2012). One of the actions detailed in the Bioeconomy Action Plan focuses specifically on promoting networks for integrated and diversified biorefineries and includes the creation of demonstration and pilot plants across Europe for the use of biomass and waste streams. Europe´s Bioeconomy Strategy was updated in 2018 to accelerate the deployment of a sustainable European bioeconomy, and its action plan includes strengthening and scaling up bio-based sectors or deploying local bioeconomies across Europe (European Commission, 2019).

The creation of demonstration and pilot plants is a crucial step before the establishment of biorefineries, including green biorefineries that produce commodities at commercial scale. During the last 10–15 years, several European countries such as Austria, Denmark, Germany, Ireland, the Netherlands and Switzerland have carried out important research in the field of green biorefineries (Mandl, 2010). Indeed, such research effort resulted in the creation of demonstration scale, pilot scale or even commercial scale green biorefinery plants. Some are summarized in Table 3.

In Austria, a pilot plant was established in 2008 for the production of amino acids, lactic acid and biogas from grass silage securing thereby decentralized operation and seasonally independent feedstock systems. The grass silage is screw pressed and then, the silage juice is treated by means of the Hybrid Process i.e. a patented process for the separation and purification of amino acids and lactic acid in two different streams. The press cake is utilized for the production of biogas in a nearby biogas plant. In Denmark, a pilot plant for the production of lysine from grass, clover or alfalfa was built in order to exploit the residual juice produced in a green crop drying plant, producing fodder pellets. The pilot plant is not operative nowadays but several research projects are currently going on with focus on the production of protein feed for pigs and poultry from green biomass. Moreover, a pilot plant and very recently a demonstration plant for the production of protein concentrates from grass, clover or alfalfa were built in Foulum, a research center of Aarhus University. The pilot plant has capacity for processing between $1-2$ tonne of fresh biomass input per hour, and the demonstration plant has a capacity of between $10-20$ tonne of fresh biomass input per hour. Germany is a pioneer country in the field of green biorefineries and a lot of research for the development and establishment of green biorefineries has been done (Alexandri et al., 2020). A green biorefinery demonstration plant for the production of fodder pellets, protein concentrates (green and white) and biogas was planned to be built in Havelland (Brandenburg), but still has not been completed. A pilot plant for the production of lactic acid by microbial fermentation of a wide variety of feedstock such as food waste, green juice, sugarcane bagasse or rice bran was built in the Leibniz Institute for Agricultural Engineering and Bioeconomy (ATB) located in Potsdam. The pilot plant has capacity for the production of 10 tonne of lactic acid per year and is framed within a general biorefinery scheme in which many different renewable and/or waste feedstock can be treated and fermented into lactic acid mainly. The lactic acid fermentation of

Table 3
Summary of green biorefinery plants in Europe and their status in 2020.

Country	Feedstock	Scale / Status	Green juice utilization	Press cake utilization	Main products	References
Austria	Silage from grassland or from clover-enriched grass	Demonstration scale plant established in 2008. Actual status unknown.	*Hybrid process* for production of amino acids and lactic acid	Feedstock for biogas plant	- Amino acids - Lactic acid - Biogas	Ecker et al., 2012; Koschuh and Kromus, 2009; Kromus et al., 2004
Denmark	Italian ryegrass, clover or alfalfa	Not operative	Lactic acid fermentation for L-lysine production	Thermal drying for fodder pellets	- L-lysine - Fodder pellets	Thomsen et al., 2004
Denmark	Grass, clover, ryegrass or alfalfa	Pilot scale plant established in 2015 1 − 2 tonne fresh biomass per hour	Precipitation of proteins by heat coagulation	Ensiled for ruminants forage	- Protein-rich feed - Press cake forage - Biogas	Corona et al., 2018; DCA, 2017
Denmark	Grass, clover ryegrass or alfalfa	Demonstration scale plant established in 2019 10 − 20 tonne fresh biomass per hour	Precipitation of proteins by heat coagulation	Ensiled for ruminants forage	- Protein-rich feed - Press cake forage - Biogas	DCA, 2019
Germany ATB, Potsdam	Alfalfa or clover grass	Pilot plant 10 t of lactic acid per year	Lactic acid fermentation with *Bacillus coagulans*		- L(+)-Lactic acid: PLA for bioplastic	Dietz et al., 2016; Papendiek and Venus, 2014
Germany Havelland	Grass, clover or alfalfa	Demonstration scale 20,000 tonne per year Not operative	Precipitation of proteins; separation of green and white proteins Residual liquid into biogas	Thermal drying for fodder pellets	- Green proteins: feed - White proteins: food or cosmetics - Fodder pellets - Biogas	Kamm et al., 2016, 2010, 2009
Netherlands Grassa B.V.	Grass	Mobile pilot installation built in 2011 1 − 5 tonne fresh biomass per hour	Precipitation of proteins by heat coagulation Extraction of P and other minerals		- Protein concentrate: animal feed - Mineral fertilizer	Grassa, 2018
Switzerland Biowert (Germany)	Meadow grass	Commercial scale plant	Extraction of proteins by ultrafiltration and reverse osmosis	Extraction of fibers and production of plastic Process unknown	- Proteins - Insulation material - Plastic - Fertilizer - Biogas	Biowert Industrie GmbH, 2018
Switzerland Gramitherm®	Grass silage	Commercial scale plant	Biogas plant or production of animal feed (under development)	Extraction of fibers Process unknown	- Thermal insulating boards - Biogas	Gramitherm® I.P. and Technology, 2018

green juice by a *Bacillus coagulans* strain results in the production of L(+)-lactic acid that is suitable for bio-plastic production (PLA, poly-lactic acid). A lot of research in the field of green biorefinery is being carried out in the Netherlands, where there is strong dependency on soybean meal imports for feeding monogastric animals. In this context, several companies involved in a collaborative research built the first mobile pilot grass refinery in 2011. Based on this collaboration, the company Grassa B.V. was founded in 2014. The mobile pilot refinery utilizes grass as main feedstock for the production of protein feed for pigs and poultry, but other crops and crop residues like green manure, alfalfa, potato, rood and beet leaves can be processed as well. Approximately 50 kg of protein concentrate can be produced from 1 tonne of fresh grass.

Some companies have established commercial scale green biorefineries, and their products are already commercialized. One example is the company BIOWERT, which has a grass biorefinery plant located in Odenwald (Germany) even though the research, marketing and management of the company takes place in Switzerland. The biorefinery plant is provided with local meadow grass for the extraction of proteins by ultrafiltration and reverse osmosis into a protein product (AgriProtBW), which can be utilized as raw material for production of flavors and cosmetic products or as animal protein feed. BIOWERT produces insulation material from the grass fibers (AgriCellBW), plastic for the automotive industry (AgriPlastBW), and natural fertilizer (AgriFerBW). Another Swiss company called Gramitherm® is producing thermal insulating boards from the fibers extracted from grass silage. The silage juice is currently utilized for biogas production but the production of a concentrate for animal feed is under development.

6. Conclusions

Given the increasing demand for meat and other animal products and an increasing interest in organic products, the extraction of proteins from leaves represents a promising solution for producing protein-rich feeds for monogastric animals in a more sustainable manner while decreasing the strong dependency for soybean and soybean meal import in Europe. Even though a lot of research was carried out in this field previously, recent technological developments and biotechnological advances may facilitate the industrial implementation of leaf protein extraction processes. Integrating the production of LPC within green biorefineries will encourage the establishment of production facilities and promote the development of a more sustainable and self-sufficient agriculture and bioeconomy in Europe. Among the main challenges are the logistics, as the production of LPC requires freshly harvested material, and the variation of the LPC due to different feedstock plant material both in terms of species/variety and in terms of plant development stage. Another challenge is to ensure LPC with high feeding value in terms of high protein content with high digestibility, which is a challenge that can be overcome depending on the choice of biorefining method. Finally, commercial production needs to be economically viable. Several calculations have shown that this can be the case, especially for production of organic LPC if the residual streams also are utilized for products, e.g. as ruminant feed, biogas and fertilizer.

Author statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influcenced its outcome.

We confirm that the manuscript has been read and approved by both authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by both of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property.

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