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Diversifying the utility and species composition of Nordic forage systems

New fractionation methods and novel native legume species

Brooke Micke

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Brooke Micke
Faculty of Natural Resources and Agricultural Sciences
Department of Crop Production Ecology
Umeå

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Cover: A biodiverse ley containing *Phleum pratense*, *Vicia sepium*, *Vicia cracca*, and *Lathyrus pratensis* established at Röbäcksdalen Field Research Station for the field trial used in paper IV.

(photo: B. Micke)

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Swedish University of Agricultural Sciences, Department of Crop Production Ecology, Umeå, Sweden

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Diversifying the utility and species composition of Nordic forage systems. New fractionation methods and novel legume species.

Abstract

Leys, a temporary grassland grown as part of a crop rotation, represent an essential component of agricultural production in the Nordic countries. This thesis explores methods to diversify the utility of feed produced from leys through fractionation, as well as increase the diversity of legume species within the stand. Two fractionation methods, press juicing and leaf stripping, were assessed in tandem on pure legume stands, with juicing producing a biorefined fraction with a lower neutral detergent fiber (aNDFom) content than leaf stripping. Both methods demonstrated variability in crude protein (CP) and biomass allocation to the resulting fractions, likely influenced by stand characteristics and machine functionality. Leaf stripping fractionation was tested on mixed grass/legume stands and produced a biorefined fraction with a higher CP content and digestibility and a lower aNDFom concentration than the mixed sward if harvested conventionally. In order to increase the biodiversity of leys, wild legume species native to northern Sweden were evaluated for their agronomic potential using botanical resources, such as herbaria, floras, and databases. Following the selection process, seven wild legume species were selected, with early flowering species being preferred due to their ability to provide floral resources early in the season, when pollinator resources are sparse in agricultural landscapes. Four of these seven species were subsequently studied when grown in mixed stands with timothy grass. Two of the species, *Vicia sepium* and *Vicia cracca* maintained promising nutritive value across multiple harvesting frequencies, though additional years of data collection are needed to assess their potential for inclusion in leys.

Keywords: forages, leys, fractionation, native species, nutritive value, yield, Fabaceae, sustainable agriculture, agrobiodiversity

Diversifiering av användning och sammansättning i vallar från Nordiska odlingssystem. Nya fraktioneringsmetoder och vilda baljväxtarter.

Sammanfattning

Vallen utgör en väsentlig del av jordbruksproduktionen i de nordiska länderna. Denna avhandling utforskar metoder för att diversifiera användbarheten av foder som produceras från vall genom fraktionering, samt genom att öka mångfalden av baljväxtarter i beståndet. Två fraktioneringsmetoder, juicepressning och bladskörd, utvärderades parallellt på rena baljväxtbestånd, där pressningen gav en bioraffinerad fraktion med lägre fiberhalt än bladskörden. Båda metoderna visade på variation i biomassa- och proteinallokering till de resulterande fraktionerna, troligen påverkat av beståndsegenskaper och maskinfunktionalitet. Bladskörd testades också på blandade gräs/baljväxtbestånd och där den bioraffinerade fraktionen hade högre proteinhalt och smältbarhet och lägre fiberkoncentration än hela vallgrödan. För att öka vallarnas biologiska mångfald utvärderades vilda baljväxtarter hemmahörande i norra Sverige för sin agronomiska potential med hjälp av botaniska resurser, såsom herbarier, floror och databaser. Efter urvalsprocessen valdes sju vilda baljväxtarter ut, där tidigt blommande arter föredrogs på grund av deras förmåga att tillhandahålla blommor tidigt på säsongen när resurserna för pollinatörer är sparsamma i jordbrukslandskapet. Fyra av dessa sju arter studerades sedan när de odlades i blandade bestånd med timotej. Två av arterna, *Vicia sepium* och *Vicia cracca* bibehöll ett lovande näringsvärde över flera skördefrekvenser, även om ytterligare år av datainsamling krävs för att bedöma deras potential för inkludering i vall.

Nyckelord: Grovfoder, vall, fraktionering, inhemska arter, näringsvärde, avkastning, Fabaceae, hållbart jordbruk, biologisk mångfald

Dedication

To Emil

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Micke B., S. Adler, J. Forkman, and D. Parsons (2023). Production and nutrient composition of forage legume fractions produced by juicing and leaf stripping (submitted).

II. Micke B., S. Bergqvist, S. Adler, J. Morel, and D. Parsons (2023). Fractionation of mixed grass and clover stands using a leaf stripper. *Grass and Forage Science*, 1-12.

III. Micke B. and D. Parsons (2023). Using botanical resources to select wild forage legumes for domestication in temperate grassland agricultural systems. *Agronomy for Sustainable Development*, 43 (1), 1-11.

IV. Micke B., A.M. Gustavsson, J. Forkman, S. Adler, and D. Parsons. Nutritive value and agronomic potential of wild forage legume species grown in a grassland agricultural system (manuscript).

The contribution of Brooke Micke to the papers included in this thesis was as follows:

I. Collected, prepared, and analysed the data with co-authors and main supervisor. Wrote the manuscript with input from co-authors and main supervisor.

II. Collected, prepared, and analysed the data with co-authors and main supervisor. Wrote the manuscript with contributions and input from the co-authors and main supervisor.

III. Conceptualized project idea and acquired funding for the work with main supervisor. Planned the study and collected, prepared, and analysed the data. Wrote the manuscript with input from main supervisor.

IV. Conceptualized project idea and acquired funding for the work with main supervisor. Planned the study along with main supervisor. Collected, prepared, and analysed the data with co-authors and main supervisor. Wrote the manuscript with input from co-authors and main supervisor.

Abbreviations

aNDFom	Amylase treated, ash-free neutral detergent fiber
CF	Clover fraction
CP	Crude protein
DM	Dry matter
GF	Grass fraction
ha	Hectare
ILDIS	International Legume Database and Information Service
LSF	Leaf stripper fraction
N	Nitrogen
OMD	Organic matter digestibility
PAPGI	The Perennial Agriculture Project Global Inventory
R^2	Coefficient of determination
RF	Residual fraction
UME	Umeå University Herbarium
VOS	Rumen degradable organic matter

1. Introduction

1.1 Grassland forage production: leys in focus

Grasslands play a major role in the agricultural landscape of Europe, covering over one third of the EU's agricultural area (Velthof et al., 2014). Large variability exists in grassland utility, management intensity, and species composition, with a major distinction made between agriculturally improved and semi-natural grasslands. Agriculturally improved grasslands provide the basis for the production of forage in Europe, through the grazing and/or harvesting of permanent and temporary grasslands. The European Commission differentiated between grassland categories used for farm structure surveys, defining temporary grasslands as "grass plants for grazing, hay or silage included as a part of a normal crop rotation, lasting at least one crop year and less than five years, sown with grass or grass mixtures" (Regulation No 1200/2009, 2009). Leys, a temporary grassland comprised of forages grown in rotation with annual crops, represent an essential improved temporary grassland in regions important for ruminant livestock production (Martin et al., 2020).

Leys are generally composed of mixed swards of grasses and forage legumes, with forbs included as minor components in some leys. Leys can be further categorized by their utility, managed for grazing or for the production of silage. As this thesis focuses on machine-harvested leys, which produce forage for ensiling, all further reference to leys will focus on those cultivated for harvest. The composition of ley swards varies greatly between regions and individual farms. The most prevalent grass species in northern Europe include timothy (*Phleum pratense* L.), meadow fescue (*Festuca pratensis* Huds.), perennial ryegrass (*Lolium perenne* L.), and Italian ryegrass (*Lolium multiflorum* L.), among others (Frankow-Lindberg, 2017). The list of common legume species is more limited, with the majority of leys utilizing red clover (*Trifolium pratense* L.), lucerne (*Medicago sativa* L.), or white clover (*Trifolium repens* L.). Though less widely used, birds-foot trefoil (*Lotus corniculatus* L.), alsike clover (*Trifolium hybridum* L.), Italian sainfoin (*Hedysarum coronarium* L.), and oriental goat's rue (*Galega orientalis* Lam.) represent alternative forage legume options for ley seed mixtures (Halling et al., 2001; Howieson et al., 2008). In the case of herbal leys, forb species such as, dandelion (*Taraxacum officinale* F.H. Wigg.), plantain (*Plantago lanceolata* L.), or common chicory (*Cichorium intybus* L.) are commonly included with the grass and legume components of the mixture (Peeters et al., 2019). Mixtures of these functional plant groups provide agronomic benefits in comparison to grass monocultures, including increased yields, higher nutritive value, and decreased inputs (Peyraud et al., 2009). In addition to these agronomic benefits, leys also have the potential to contribute numerous environmental services. Diverse species swards that include legumes and other deep-rooting perennials have been show to improve soil fertility and structure, increase above and below ground biodiversity, decrease dependence on nitrogen fertilizer, and provide both habitat and food resources for a variety of organisms (Bianchi et al., 2013; Cooledge et al., 2022; Martin et al., 2020).

1.1.1 Ley production in northern Sweden

The Swedish word for ley, "vall", was introduced in the 18th century. During this time, natural meadows were progressively replaced by artificially sown leys that were included in rotation with cereal production in order to improve soil nutrient composition (Tuvesson, 2001). Today, leys still make up an essential component of Swedish agriculture, particularly in the north. In the four northernmost provinces, Västernorrland, Jämtland, Västerbotten, and Norrbotten, leys occupied between 60-70% of arable land in 2022, a considerably higher proportion than seen on the national scale (Figure 1). Climatic conditions are highly variable between the northern and southern regions of Sweden, with the south having upwards of 100 additional growing season days compared to the north (Jordbruksverket, 2009). This large climatic scale results in a crop distribution disparity throughout the country, with horticultural crops, bread grains, and oilseeds localized to the south and forages and some cereals representing the majority of production in the north. The climate of northern Sweden is ideal for the production of high quality forage. Summers in the north provide a practically unlimited amount of sunlight for photosynthesis, with up to 24 hours of daylight in regions above the Arctic Circle. These long days enable high growth productivity, while also maintaining a low fiber content. Additionally, the mild summer temperatures in the region reduce the accumulation of fibre, resulting in forage with a high nutritive value for ruminants (Ericson, 2018). The high quality forage produced in the north comprises an essential feed component for the estimated 30,000 dairy cows in the four northernmost provinces (Jordbruksverket, 2022).

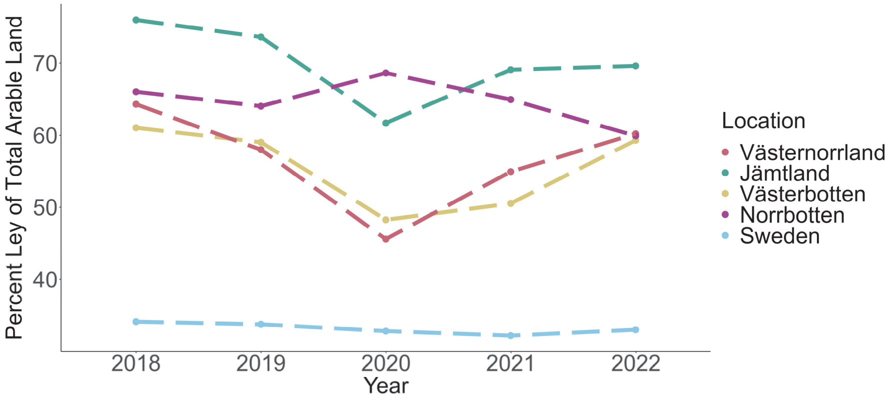

Figure 1. The land area of leys expressed as the percentage of total arable land for the four northernmost provinces in Sweden (Västernorrland, Jämtland, Västerbotten, and Norrbotten) and for the entirety of Sweden. Data for the figure was acquired from the Jordbruksverket Statistic Database (2022).

Leys in the north are typically composed of swards of red clover in mixture with forage grasses, most commonly timothy. Recommended seed mixtures for mixed grass-clover swards in Sweden include timothy and red clover at sowing rates of 15 kg ha^{-1} and 5-7 kg ha^{-1}, respectively (Frankow-Lindberg, 1990). Generally, leys in northern Sweden have a duration of three to four years before being resown, though durations of up to 8 years have been observed (Ericson, 2018). Harvest frequency in the region ranges dependent on latitude, with one- to three-cut systems utilized to achieve variable nutritive value and yield targets (Frankow-Lindberg, 2017). The limited number of harvests in the north compared to the south is preferred due to an increased frequency of winter kill at higher latitudes when additional harvests are taken late in the season (Andersson, 1997; Halling, 1994). Harvest regime has a major impact on the nutritive value of the sward, with three-cut systems resulting in forage with a higher digestibility and lower fiber content. Alternatively, two-cut systems have been shown to produce higher total yields (Gunnarsson et al., 2014; Tuvesson, 1986, 2001).

1.1.2 Common issues in Swedish ley production

The preferred management strategy utilized in Sweden poses problems for the consistent harvest of high quality forage throughout the entire lifespan of the ley. Diseases such as clover rot and root rot negatively impact the persistence of red clover, particularly hindering their winter survival (Pulli et al., 1996). Clover rot is the result of the pathogen *Sclerotinia trifoliorum* Erikss., a soil borne ascomycete capable of infecting leguminous plants (Eriksson, 1880). The root rot disease complex is comprised of pathogens within the genus *Fusarium* (Ylimäki, 1967). As fungi within this complex are generally weak pathogens, damage typically occurs with increased plant age when plants have previously been injured (Öhberg, 2008). The damage caused by clover and root rot is generally most prevalent during winter dormancy of red clover, resulting in a high proportion of plant death in late winter and spring (Öhberg, 2008). Tetraploid cultivars with late flowering have been shown to be more resistant to clover rot, though serious yield losses attributed to clover rot are still common in northern Sweden (Öhberg et al., 2008). This is likely due to the more aggressive strains of *Sclerotinia trifoliorum* that are found in the north (Öhberg et al., 2005). Fungicides offer protection against clover and root rot, but are not currently allowed in Sweden (Nan et al., 1991; Raynal et al., 1991). Issues with persistence become more complicated when considering root rot, as tetraploid varieties bred for clover rot resistance have been shown to be more susceptible to root rot (Öhberg, 2008). Root rot occurrence becomes more prevalent in later years of the ley, making it a major driver of red clover losses in the third year of production and onwards (Ylimäki, 1967). Alternative forage legume species that are more resistant to clover and root rot could provide an additional legume component to the ley in later years of production, following the loss of red clover.

The nutritive value of forage is heavily dependent on maturity stage at the time of harvest (Figure 2), with a changing leaf:stem ratio responsible for a decreasing nutritive value with increased maturity (Buxton, 1996). Leaves of forage legumes and grasses differ in nutritive value compared to the stems, with leaves having higher protein concentrations and a lower fiber content (Solati et al., 2018). This disparity in protein and fiber is the result of the high proportion of photosynthetic machinery and a lower cell wall content present in the leaves (Fiorentini & Galoppini, 1983). Due to shifting nutritive value of forages, identifying the appropriate harvest date corresponding to the proper maturity stage becomes essential to achieve forage with the targeted nutritive value. Without harvest maturity consistency, the nutritive value of forage-based feed can experience high levels of variability, thus requiring the addition of concentrates to maintain livestock productivity (Morel et al., 2022). Selection of the optimal harvest window for multi-cut leys presents challenges, as current methods used by farmers utilize growing degree day estimations that are only suitable for selecting the date of the first harvest (Ragnmark, 2012). Alternative methods for harvest day selection, such as the use of field spectrometers to estimate nutritive value, show promise, but the cost of these instruments would limit their widespread integration (Morel et al., 2022; Zhou et al., 2019). Post-harvest processing of forage presents an opportunity to increase its nutritive value, minimize the influence of harvest maturity stage on its feed potential, and expand its utility beyond ruminant production.

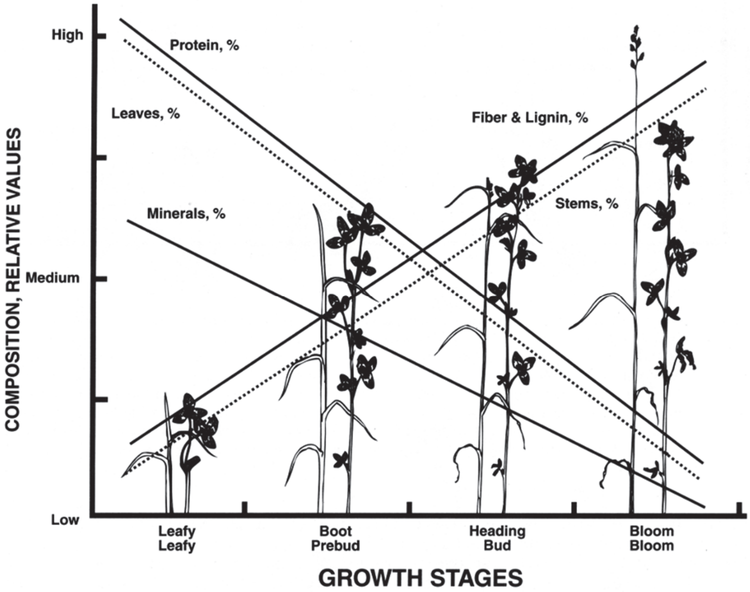

Figure 2. The nutritive value of perennial forage grasses and legumes shift with increasing maturity stage. The decrease in protein and increase in fiber with advancing maturity is heavily influenced by the changing leaf:stem ratio (White & Wolf, 2009).

In addition to the influence of harvest date on nutritive value, ley forage is also negatively impacted by the poor persistence of red clover. As the clover component of ley stands decreases, so does it nutritive value and yield stability (Marshall et al., 2017). In later years of production, the sward composition shifts drastically, with the loss of clover leading to stands solely composed of grass and weeds. The lower protein and higher fiber concentrations present in grasses compared to legumes results in forage of lower nutritive value (Buxton, 1996; Nilsdotter-Linde et al., 2002). Additionally, yield stability of the grass stand is highly dependent on the application of nitrogen fertilizer, thus increasing input costs and negative environmental impacts of ley production (Frankow-Lindberg, 2017). This variability in nutritive value and yield caused by the loss of red clover in the sward could potentially be remedied by the inclusion of alternative legume species with superior persistence. These species could provide a legume component and contribute biologically fixed nitrogen to the stand in later years of production.

In northern Sweden, harvest decisions for mixed leys are generally made according to the phenological development of the dominant grass, timothy

(Gustavsson, 2011). This commonly means that leys are harvested prior to the flowering of red clover, particularly for the first cut of the season. Though harvesting at this early phenological stage is preferable for forage quality, it limits the availability of food resources for local pollinators. The decline of pollinators has been well documented globally, with the increase in cultivated land often cited as a major contributor (Aizen et al., 2019; Gallai et al., 2009). Increased agrobiodiversity can help alleviate the negative impacts of agricultural land on pollinators by providing a range of food resources with high temporal diversity (Wratten et al., 2012). Grasslands, such as mixed leys, are capable of housing high levels of plant diversity representing various functional groups. When managed with the intention to increase pollinator resources, agrobiodiverse leys could contribute nectar and pollen early in the season when food source availability is inadequate (Johansen et al., 2019).

1.1.3 Altering the nutritive value of forage for monogastrics

Though forage harvested from leys plays an essential role in the diet of ruminants, its utility is limited for monogastrics (Kass et al., 1980). Monogastrics have relatively strict feed demands, requiring feed sources high in protein with a well-balanced amino acid composition. Additionally, monogastrics have limited ability to digest unprocessed fibers, thus limiting the inclusion of feeds with a high fiber content in their diets (Laudadio et al., 2014). The production of suitable protein-rich feed is not well developed in northern Europe, requiring the import of soybean-based feed products to support the livestock sector’s protein-feed requirements (Häusling, 2011). Sweden alone imported roughly 250,000 tons of soybean products in 2021 (Food and Agriculture Organization of the United Nations, 2023). Shifting harvest windows and selecting harvest frequencies for high quality forage are not sufficient methods for producing forage with suitable nutrient composition for monogastrics. Additional processing of forage, however, has been shown to produce protein-rich feed products with a nutrient profile congruous to monogastrics requirements (Damborg et al., 2020; Jørgensen et al., 2022; Renaudeau et al., 2022; Stødkilde et al., 2018). The fractionation of forage plants could enable the diverse usage of locally produced feed to supply the monogastric livestock sector with sustainable alternatives to imported soybean.

1.1.4 Increasing the persistence and biodiversity of leys

The challenge of maintaining stable yield and quality of leys in later years of production has not been resolved despite continued efforts to increase the persistence of red clover cultivars (Marshall et al., 2017; Öhberg, 2008). Though its poor persistence impairs the overall quality and yield of leys in the long-term, its superior nutritive value and biomass production in early years warrant its importance for forage production. The productivity of red clover can be attributed to centuries of breeding efforts that fine-tuned its agronomic potential (Abberton & Marshall, 2005; Nay et al., 2023). Due to this, the likelihood of identifying an alternative to red clover with comparable yield and nutritive value, and the capability for production in the Nordic climate is unlikely. In lieu of replacing red clover, the inclusion of wild legume species with increased persistence as minor components of leys could assist in maintaining nutritive value and yield of the sward in later years of production. Though the pathogen susceptibility of wild legume species is not well understood, the inclusion of species capable of rhizomatous growth could ensure population stability throughout the lifespan of the ley. In addition to improving the persistence of leys, the inclusion of wild legume species would increase the system's agrobiodiversity and subsequently supply ecosystem services to improve the sustainability of forage production (Bianchi et al., 2013).

1.2 Forage fractionation

Perennial, grassland forage crops are a sustainable biomass source, particularly in northern Europe where climatic conditions are ideal for high productivity (Manevski et al., 2018; Pugesgaard et al., 2015). As forages are an unsuitable feed source for monogastrics, additional processing is required to broaden their utility (Laudadio et al., 2014). Fractionation of forage presents an opportunity to exploit the productivity of grassland crops for the local production of protein-rich feed suitable for monogastrics, as well as multiple co-products such as ruminant feed, biomaterials, or various bioenergy sources (Jørgensen et al., 2022; Mandl, 2010). Forage fractionation enables the allocation of chemical components from the green biomass into multiple fractions with targeted nutrient compositions. Various forage fractionation techniques have been developed, including sieving, pin milling, air classification, twin screw press juicing, and leaf stripping

(Damborg et al., 2020; Laudadio et al., 2014; Liebhardt et al., 2022; Wu & Nichols, 2005). These fractionation methods all aim to separate the forage into a protein-rich fraction suitable for monogastrics and a fiber-rich co-product with potential to serve as a feed source for ruminants (Damborg et al., 2018; Laudadio et al., 2014; Renaudeau et al., 2022; Stødkilde et al., 2019).

The term green biorefinery is typically used to describe the processing of grassland biomass into a range of economically relevant products and energy sources (Cherubini et al., 2009). The multi-product approach of green biorefinery ensures financial security to cover the costs of biomass production and the necessary processing (Mandl, 2010). The chemical composition of the fresh forage and its subsequent preservation are key in determining the range of potential products rendered (McEniy & O' Kiely, 2014). The essential chemical components of forage plants to consider in biorefinery are the cell walls and the cell contents. Structural components, such as cellulose, hemicellulose, and lignin, comprise the majority of the cell wall and are responsible for ensuring the plant's structural integrity (Wilson, 1993). Forage cell contents are composed of proteins, sugars, and lipids, of which proteins are the main focus for biorefinery (Fiorentini & Galoppini, 1983). The most abundant of the proteins found in the cell content is ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco). Rubisco accounts for over half of the protein found in leaves, as a result of its importance in the photosynthetic process (Jensen & Bahr, 1977; Singer et al., 1952). The proportion of cell wall to cell content varies between plant structures, with the stem containing larger amounts of cell wall compared to the leaves (Fiorentini & Galoppini, 1983). Cell wall to cell content ratio is also dependent on the maturity stage of the plant, as cell wall proportion increases with advancing maturity (Buxton, 1996). The cell wall proportion of legume stems are subject to greater change based on plant maturity than the leaves, primarily due to stem thickening as the plant matures (Wilson, 1993). The primary objective of green biorefinery fractionation is to separate the fibrous cell wall components from the nutrient-rich cell contents and consolidate the majority of the available protein into a single fraction.

1.2.1 Screw press juicing

The standard fractionation method used in green biorefinery is mechanical maceration, in which grassland biomass is separated by a screw press juicer

(McEniy & O’ Kiely, 2014). Following the press juicing of forages, two fractions are created, the protein-rich juice and the fiber-rich press cake/pulp (Figure 3). The raw press juice is not suitable for direct feeding to monogastrics without further processing, due to the high water content and elevated concentrations of minerals and polyphenol oxidases (Chiesa & Gnansounou, 2011). Additionally, the raw juice has a limited shelf life, thus requiring preservation treatment. Heat coagulation or acid precipitation can be utilized to extract the soluble protein from the press juice (de Fremery et al., 1973; Pirie, 1969). The resulting protein concentrate can then be preserved to maintain the nutritive value and ensure stability, while the residual brown juice has potential as a fertilizer or as a substrate for energy production (Bruins & Sanders, 2012; Morrison & Pirie, 1961; Santamaría-Fernández & Lübeck, 2020; Worgan & Wilkins, 1977). Preservation of the protein concentrate is most commonly accomplished through drying, with freeze-drying achieving superior results to air-drying and heat treatment (Chowdhury et al., 2018; R. E. Miller et al., 1972; Morrison & Pirie, 1961). Previous studies have demonstrated the potential of the protein concentrate as an protein feed source for monogastrics (Laudadio et al., 2014; Renaudeau et al., 2022; Stødkilde et al., 2018).

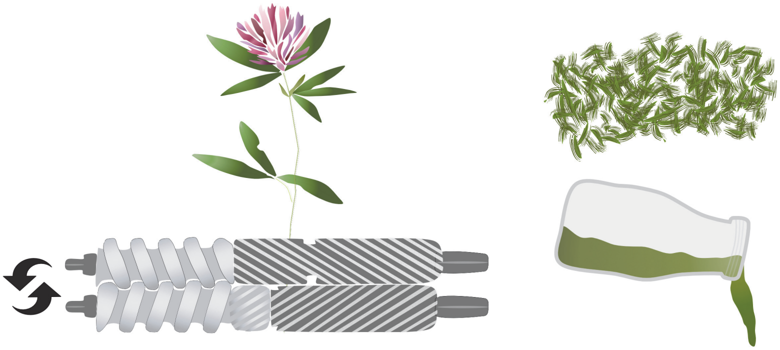

Figure 3. Illustration of fractionation through twin-screw press juicing and the resulting juice and pulp fractions. Illustration: Brooke Micke.

Nearly half of the protein from the raw forage is allocated to the pulp fraction, as a significant amount of the available protein is fiber bound (Morrison & Pirie, 1961). Though the pulp is not the targeted product of juicing, its utilization ensures both the economic viability of the production system, as well as the sustainable use of all produced biomass. Ensiling of the pulp can ensure the longevity of its use outside of the production season

(Damborg et al., 2020; Jørgensen et al., 2022). The nutritive value of the pulp fraction has been shown to be comparable to that of the fresh forage, indicating its potential as a feed source for ruminants (Damborg et al., 2018). The produced juice and pulp fractions can also be further processed for the creation of biofuels, such as bioethanol and biogas (Neureiter et al., 2004; Weiland, 2010).

1.2.2 Leaf stripping

The distribution of cell wall and cell content throughout the plant presents an opportunity for fractionation during harvest, as opposed to the post-harvest fractionation methods generally used. Forage leaves contain lower proportions of cell wall compared to the stems, as well as significant amounts of soluble protein due to the high concentrations of rubisco as part of the photosynthetic machinery (Fiorentini & Galoppini, 1983). Previous studies have shown that forage legume leaves contain considerably higher amounts of the plant's extractable protein than the stems (Hakl et al., 2016; Solati et al., 2018). By capitalizing on the allocation of nutrients within different plant organs, leaf stripping enables the fractionation of forages during the harvest process. Fractionation through leaf stripping is achieved by utilizing specialized or modified harvest machinery that removes only the leaves and soft, upper stem fragments, leaving the fibrous forage stems behind (Figure 4). The potential of a variety of leaf stripper designs has been demonstrated, from specially designed machinery with rotating tines that remove leaves to green bean harvesters modified to strip the leaves from the stems (Liebhardt et al., 2022; Shinners et al., 2007). The leaf fraction has been shown to have potential as an energy feed source for monogastrics, due to the high concentration of easily digestible carbohydrates (Renaudeau et al., 2022). The preservation of the leaf fraction through drying or ensiling can ensure the availability of protein-rich feed throughout the year (Digman et al., 2013; Sikora et al., 2019, 2021). The remaining stems can subsequently be harvested using conventional harvest machinery. With a higher fiber concentration, the stem fraction may be suited as ruminant feed following ensiling. Compared to press juicing, leaf stripping is a far less studied fractionation technique, with the majority of studies focusing solely on pure stands of lucerne (Andrzejewska et al., 2020; Currence & Buchele, 1967; Hakl et al., 2016; Sikora et al., 2019). The integration of leaf stripping in northern Europe is reliant on its ability to fractionate mixed stands, requiring additional work to understand the potential of the method on multispecies swards.

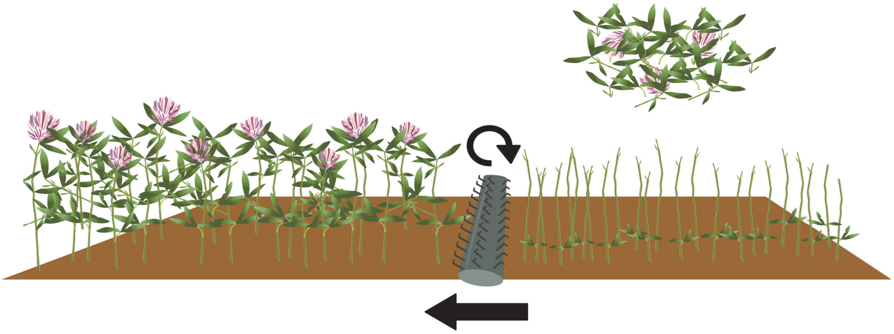

Figure 4. Illustration of fractionation using a leaf stripper and the resulting leaf and stem fractions. Illustration: Brooke Micke.

1.3 Diversity in forage legumes

With nearly 20,000 species, Fabaceae is the third largest family of flowering plants (Azani et al., 2017). This immense collection of biodiversity, however, is not adequately represented in agriculture. Only 65 species from the legume family are considered commercially important, 50 of which are forage legumes (Howieson et al., 2008; Schlautman et al., 2018). This gap indicates that additional legume species with unique adaptations to their environment may have agronomic potential and thus should be considered for inclusion in agricultural systems. Investigation into wild and underutilized legume species as forages and seed crops is not new, with many researchers highlighting their agricultural potential (Bhat & Karim, 2009; Howieson et al., 2008; Muir et al., 2005; Schlautman et al., 2018; Zhang et al., 2019). The importance of forage legumes as animal feed has been apparent throughout history, with the cultivation of lucerne as the first forage crop over 9,000 years ago (Ghaleb et al., 2021). Excluding lucerne, red clover, white clover, alsike clover, bird's-foot trefoil, and Italian sainfoin, knowledge on the agronomic and physiological characteristics of temperate perennial forage legumes is sparse (Howieson et al., 2008). Any potential for the inclusion of wild forage legume species in agricultural systems relies on further study of their agronomically important traits.

1.3.1 Selection of new species for domestication

In recent years, there has been an increased interest in the study and conservation of crop wild relatives for their potential genetic resources. Crop wild relatives can be defined as: “a wild plant taxon that has an indirect use derived from its relatively close genetic relationship to a crop” (Maxted et al., 2006). With such a broad classification of crop wild relatives, it becomes important to determine which wild species are of most interest so that conservation and research efforts can be better focused. Generally, wild plant species are selected as crop wild relatives through their taxonomic proximity to current crops (Fitzgerald et al., 2019). Though this method is well suited to the use of wild species as gene donors for crop modification, it excludes species with agronomic potential that could benefit agricultural systems through their domestication.

Botanical resources, such as herbaria, databases, and floras, provide morphology, phenology, and taxonomy data on nearly every species of flowering plants (Kattge et al., 2020; Missouri Botanical Garden., n.d.; Molina-Venegas et al., 2021). These resources, compiled over centuries, are continually expanding due to the efforts of over 3000 botanical gardens and herbaria (A. J. Miller et al., 2015). With over 390 million herbarium specimens (Figure 5) globally, these herbaria provide an ideal opportunity to select crop wild relatives for domestication based on traits of interest (Thiers, 2016). Due to the large digitisation effort that has been in effect over the last 30 years, these herbarium specimens have become more accessible to researchers around the world (Tulig et al., 2012). This large scale digitisation effort has allowed for novel utilization of herbarium specimens in many areas of research, such as global change biology, phytochemistry, and agronomy (Cook et al., 2021; Meineke et al., 2018; Willis et al., 2017). In recent years, prominent botanical institutions, such as the Missouri Botanical Garden and the Royal Botanic Garden Kew, have initiated projects that demonstrate the potential of integrating botanical knowledge in the identification and conservation of crop wild relatives (Ciotir et al., 2019; Dempewolf et al., 2014). The Perennial Agriculture Project Global Inventory (PAPGI), a collaboration between Saint Louis University, The Missouri Botanical Garden, and The Land Institute, best demonstrates the potential of using botanical knowledge in the advancement of agrobiodiversity research. PAPGI, a database embedded within Tropicos, compiles data on all perennial, wild, herbaceous species of the plant families Asteraceae, Fabaceae, and Poaceae (Ciotir et al., 2019). Through the integration of numerous botanical databases, PAPGI utilizes botanical knowledge from herbaria, gene banks, libraries, and scientific publications to create a checklist for plant breeders interested in the domestication of wild, perennial species from the three most agriculturally relevant plant families. The database combines diverse information on taxonomy, growth, ecology, reproductive biology, genetics, economic use, and toxicity and assembles it into a single site for ease of use. PAPGI's novel approach demonstrates the potential for advancement in crop wild relative research through the exploration of botanical resources.

Namn Vicia cracca.
Kråkvicker
Klass 17
Familj Leguminosæ
Växtställe Vägkant, Utby
Utbyden 12/6 19 46
Inga-Britt Johansson

PLANTÆ SCANDINAVIÆ.
Namn: Vicia sepium
Häckvicker
Klass: 17
Ordning:
Nat. fam.: Leguminosæ
Växtställe: [illegible] Bäck (Västerg)
den 29 maj 1935
P. Vallborg Johansson

Figure 5. Image of herbarium specimens of *Vicia cracca* L. (left) and *Vicia sepium* L. (right) from the Umeå University Herbarium. Photo: Brooke Micke.

1.3.2 Agronomic potential of wild forage legumes

To select wild legume species with the potential for domestication as forages, morphological traits such as habit, plant height, and growth duration must fit the management strategy of the intended cropping system. The ethnobotanical history of the species is also of interest, as species with a historical use as forage may have great potential for use in forage systems today (Frawley et al., 2020; Schlautman et al., 2018). Additionally, the cultivation potential of the wild legume species must be evaluated to determine their response to management, competition potential, establishment capability, and soil type suitability. Management intensity and harvest frequency have been shown to influence the botanical composition, productivity, persistence, and nutritive value of mixed stands (Biligetu et al., 2021; Raus et al., 2012). Determining suitable management practices and harvest regimes will be an essential aspect in the integration of wild legumes in agricultural systems. As the seeds of many legume species exhibit high levels of physical dormancy, scarification techniques to overcome hardseededness must be assessed to ensure germination success (Statwick, 2016; Y. Wang & Hu, 2013). The potential for seed production will be a major hurdle to the large-scale implementation of wild legume species and work will be required to determine best methods for achieving sufficient seed yields (Boelt et al., 2015).

A greater understanding of the nutritional composition of wild legumes is required to ensure feed quality is appropriate for the intended livestock (Howieson et al., 2008). Nutritive value parameters, such as crude protein, neutral detergent fiber, and digestibility can indicate the suitability of wild legumes as feed resources in animal production (Bhat & Karim, 2009). An important concern when considering the inclusion of novel forage species in leys is their potential negative impact on the nutrition of livestock. The presence of anti-nutritional factors greatly limits the utility of forage and minimizes animal productivity (Mueller-Harvey et al., 2019; Pecetti et al., 2006; Ramteke et al., 2019). The major anti-nutritional factors of concern in forage legumes are condensed tannins, saponins, and phytoestrogens. In high concentrations, these anti-nutritional factors reduce digestibility and nutrient utilization, decrease voluntary feed uptake, and negatively impact development and reproduction (Hloucalová et al., 2016; Höjer, 2012).

A major role of forage legumes in cropping systems is the supply of biological nitrogen (Carlsson & Huss-Danell, 2003). To ensure wild legume species are capable of contributing N, it is essential to understand the specificity of their rhizobial symbioses (D. Wang et al., 2012). As nodulation is best studied in agriculturally relevant species, wild legume species will require comprehensive research into their ability to nodulate and potential N_2 fixation capacity. A study on 26 species and four subspecies of native legumes in Sweden demonstrated nodulation in all 30 taxa (Ampomah et al., 2012). These results signify that the evaluated species are capable of fixing nitrogen in their native environments. Identifying the natural rhizobial symbiont to these native legumes is essential to their agronomic success, as inoculants may need to be produced. Some work has been done to identify rhizobia of Swedish legumes, such as *Lotus corniculatus*, *Anthyllis vulneraria* L., and five *Vicia* spp, though additional work is still required (Ampomah & Huss-Danell, 2011, 2016).

1.3.3 Benefits to the inclusion of wild legumes

The inclusion of wild legumes in northern Swedish leys has great potential to improve both the system and the surrounding ecosystem. High levels of agrobiodiversity have been shown to have beneficial implications for yield, persistence, input reduction, and weed suppression in grassland agricultural systems. Diverse grassland mixtures help ensure yield stability through maintaining the persistence and productivity of the sward under variable management structures (Jing et al., 2017). This stability can be attributed to improved resource use efficiency from diverse mixtures of species and functional groups (Carlsson et al., 2017; Connolly et al., 2018). Ley persistence can also be benefited by increased legume biodiversity. It has been shown that other perennial legumes species, such as white clover and birdsfoot trefoil have greater persistence than red clover (Wallenhammar et al., 2008). The inclusion of more persistent legume species can act to increase the persistence of the system, while also reducing the need for the input of nitrogen fertilizer, particularly in the later years of production (Ericson, 2005; Slepetys, 2008). By increasing persistence and resource acquisition and reducing the dependence on fertilizer, systems with higher levels of agrobiodiversity experience lower degrees of weed invasion compared to monocultures and simplistic mixtures (Connolly et al., 2018; Frankow-Lindberg et al., 2009).

Increased biodiversity also has the potential to improve the sustainability of leys through the contribution of ecosystem services. Multi-species leys have been shown to increase resources for pollinators and other beneficial arthropods, inhibit the degradation of soil, decrease nutrient losses and the subsequent eutrophication, and decrease enteric methane emissions. Species-rich grasslands help provide arthropod-mediated ecosystem services, such as pollination, through the contribution of diverse pollen and nectar resources (Decourtye et al., 2010). The benefit of this diversity is expanded when species richness focuses on the integration of native plant species, as native plants are better adapted to the local ecosystem and can provide a temporal distribution of resources (Isaacs et al., 2009). Biodiverse, legume-focused grasslands can also minimize soil degradation through the supply of biologically fixed nitrogen and restoration of earthworm populations (Carlsson & Huss-Danell, 2003; Hallam et al., 2020). Multi-year ley rotations decrease the frequency of tillage, thus providing suitable long-term habitat for earthworms (Arai et al., 2018). This increase in earthworm populations can further assist in the restoration of soil fertility. The nitrogen supplied to the system by legumes decreases its dependence on nitrogen fertilizer (Riesinger & Herzon, 2010). This input reduction can assist in minimizing the over application of fertilizer and the subsequent nutrient pollution of neighboring aquatic ecosystems (Power, 2010; Vitousek et al., 2009). Though secondary plant metabolites can have negative implications for animal health and production in high concentrations, condensed tannins and saponins have also been shown to moderate microbial production in the rumen (Bharanidharan et al., 2018). This microbial moderation has been shown to reduce enteric methane emissions from ruminant production (Aboagye & Beauchemin, 2019; Bodas et al., 2012).

2. Aims

The overall aim of this thesis was to explore the nutritive value and yield potential of various fractionation products and novel legume species in an effort to diversify forage production in northern Sweden.

This aim was evaluated based on the following specific objectives:

I. Evaluate the nutrient composition and yield of biorefined and residual fractions obtained through juicing and leaf stripping pure stands of forage legumes in Norway and Sweden.
II. Evaluate the nutritive value and yield of biorefined and residual fractions obtained through leaf stripping mixed stands of red clover and timothy compared to the mixed sward when harvested conventionally.
III. Identify wild forage legume species native to northern Sweden and investigate their potential for inclusion in mixed leys based on key traits of interest.
IV. Investigate the nutritive value and yield of four wild forage legumes under varying harvest frequencies when grown in mixed stands with timothy.

3. Materials and methods

This thesis was based on data from field trials conducted in Umeå, Sweden and Tingvoll, Norway in the summers of 2019 and 2020 – **paper I**, as well as field trials conducted solely in Umeå, Sweden in 2021 and 2022 – **papers II and IV**. Additionally, data was collected from the Umeå University Herbarium and various online botanical databases in 2020 – **paper III**.

3.1 Paper I

Two field experiments were established in 2018, one in Tingvoll, Norway (62.92°N, 8.19 °E) and the other in Umeå, Sweden (63.81°N, 20.24°E). Plots were sown as monocultures of red clover (*Trifolium pratense* L.), alsike clover (*Trifolium hybridum* L.), blue lucerne (*Medicago sativa* L.), and yellow lucerne *(Medicago falcata* L.). Red clover cultivars Gandalf and Lars, the alsike clover cultivar Frida, and the blue lucerne cultivar Ludwig were used in both field experiments. The yellow lucerne cultivar, Karlu, was incorporated as a fifth legume taxon in the Swedish field experiment. At both sites, the experiments were established using a randomised complete block design composed of four blocks, with each block containing one plot of each forage legume cultivar for juicing and one for leaf stripping. The Norwegian experiment consisted of 32 plots, while the Swedish experiment consisted of 40 plots. Both locations managed the experiments organically, though the Norwegian experiment was sown on land with a long history of organic production.

Pre-harvest measurements of plant height, canopy height, and phenological stage were taken within two subplots for each experimental plot. Samples were also taken from each plot for botanical composition separations. Plots for fractionation by juicing were harvested using a mower harvester and a subsample was taken for subsequent juicing. Fractionation through juicing was performed using a lab-scale twin-screw press juicer (Figure 6A). The subsample of whole plant material harvested from experimental plots was juiced and the resulting juice and pulp fractions were prepared for nutrient composition analysis and DM determination. Plots for fractionation by leaf stripper were harvested using the PremAlfa Mini electric leaf stripper prototype (Figure 6B) which utilizes rotating tines to separate leaves from the stem. Subsamples of the leaf and stem fractions were taken for nutrient composition analysis and DM determination. For both harvest treatments, yield per plot was weighed and yield calculations were made based on kg DM ha^{-1}.

Figure 6. A) The Angel 7500 (Angel CO., LTD., Korea) twin-screw press juicer used for juice fractionation in paper I and the resulting juice and pulp fractions. B) The PremAlfa Mini (Alf'ing – Trust'ing, Nantes, France) electric leaf stripper used for leaf stripper fractionation in papers I and II. Photos: Brooke Micke.

The biorefined fractions, juice and leaves, and the residual fractions, pulp and stems, were analysed according to the AOAC official methods (Association of Official Analytical Chemists, 1990) for laboratory dry matter, DM (967.03), crude protein, CP (990.03), and ash (942.05). Amylase treated, ash-free neutral detergent fiber, aNDFom, was determined according to Van Soest, Robertson, and Lewis (1991) with adaptations specified in the methods of **paper I**.

Data collection at both sites was hindered due to a delay in delivery of the leaf stripper machinery. Due to this delay, leaf stripper plots were not harvested in 2019 at the Norwegian site. Leaf stripper plots at the Swedish site, however, were harvested in the third cut of the 2019 season. Data collection at the Swedish site was further disrupted following a particularly harsh winter in the region between 2019-2020. Large amounts of ice build-up on the field resulted in high winter kill rates for all cultivars included in the experiment. Two datasets were constructed using the collected data to maximize the available information for comparison of the two fractionation methods. The first dataset (2020 NO) included all data from the 2020 field season at the Norwegian site. Only data on the red clover and alsike clover cultivars were included, as lucerne establishment issues occurred in Norway. The second dataset (3rd Cut SENO) included data from the third harvest of 2019 in Sweden and the third harvest of 2020 in Norway. This dataset only included data from the red clover cultivars, due to low yields of alsike clover inhibiting its harvest in Sweden and the aforementioned lucerne establishment issues in Norway.

Linear mixed models were fitted using the SAS procedure MIXED (SAS software version 9.4, SAS Institute Inc., 2008). The output variables, CP, aNDFom, and yield, were analysed separately for the biorefined and residual fractions. The models for the 2020 NO dataset included the fixed-effects factors of cultivar, cut, and fractionation method, as well as all of their possible interactions. The models for the 3rd Cut SENO dataset included the fixed-effects factors of cultivar, location, and fractionation method, as well as the interactions. Models for both datasets included block as a random-effects factor. Tukey's method ($p < 0.05$) was used to test differences among means. Additional details on the statistical analysis can be found in **paper I**.

3.2 Paper II

Data collection for this study was executed at the Röbäcksdalen research station located in Umeå, Sweden (63.81°N, 20.24°E). Twenty sampling locations measuring four-meters in length were selected in 2021 and 2022 from areas established as mixed leys of red clover and timothy. Sampling locations were harvested throughout the entire season, with swards of different compositions, heights, and phenological stages prioritized to obtain a diverse dataset. Pre-harvest data on the height and phenological stage of red clover and timothy were taken from a subplot located in the center of the sampling area. A subplot located outside of the sampling area was hand cut for botanical composition.

The PremAlfa Mini leaf stripper prototype (Figure 6B) used in **paper I** was used to harvest the sampling area. Details on the machine settings used can be found in **paper II**. Following the harvest, the residual fraction left behind by the leaf stripper was sampled from a subplot located within the sampling area for nutritive value analysis and DM determination. In total, 40 independent samples were harvested, 20 between the 24th of June and 1st of September 2021 and 20 between the 23rd of June and 8th of September 2022. The fraction harvested by the leaf stripper was weighed and a subsample was taken for nutritive value analysis and DM determination. The botanical composition sample was hand separated into three different fractions: grass, clover, and broad-leaf weeds. The grass and clover fractions were dried for nutritive value analysis and DM determination, as the weed fractions were negligible for all samples.

Overall, four fractions were analysed for nutritive value, a grass fraction (GF), clover fraction (CF), leaf-stripper fraction (LSF), and residual fraction (RF). The nutritive value of the mixed sward was calculated based on the weighted results for the CF and GF. CP was analyzed using the Kjeldahl-N method (Nordic Committee on Food Analysis, 1976) and aNDFom was analyzed according to Chai and Udén (1998). Organic matter digestibility (OMD) was determined using the rumen degradable organic matter (VOS) method (Lindgren, 1979).

General linear mixed model procedures in PROC GLIMMIX (SAS software version 9.4, SAS Institute Inc., 2008) were analyzed for the output variables, CP, aNDFom, VOS digestibility, and ash, with plant fraction, year, and their interaction as fixed-effects factors. Tukey's method ($p < 0.05$) was used to test differences among means for significant main effects. For significant interactions, the Holm-Bonferroni method ($p < 0.05$) was used to test comparisons between the same fraction across both years and for all fractions within each year. The correlation between explanatory variables and response variables was evaluated using the Kendall correlation method, as results from a Shapiro-Wilk test determined that the data did not follow a normal distribution (R Studio software version 2022.12.0+353, R Core Team, 2022). PROC GLMSELECT (SAS software version 9.4, SAS Institute Inc., 2008) was used to build multiple regression models for predicting the nutritive value of the LSF based on pre-harvest data. Two multiple regression models were constructed for each nutritive value parameter (CP, aNDFom, VOS digestibility, and CP yield). A field model was constructed using only pre-harvest field measurements, while a full model was constructed using the pre-harvest field measurements and pre-harvest nutritive value measurements. A summary of the explanatory variables can be found in **paper II**. Two datasets were used to construct the aforementioned models, one with all explanatory variables and another limited to the explanatory variables which demonstrated moderate or strong correlation to the model's response variable. The STEPWISE option was used for variable selection, with the PRESS statistic used as the criterion. The adjusted r-squared criterion (R^2) was used for reporting of results.

3.3 Paper III

A list of herbarium specimens of all Fabaceae species found in the six northernmost faunistic provinces of Sweden was extracted from the Swedish Virtual Herbarium (http://herbarium.emg.umu.se/), resulting in 79 species representing 25 genera. A taxonomic validity inquiry was executed using the Tropicos database (https://www.tropicos.org) to ensure all species names were still valid. As four of the identified species had been reclassified as subspecies or synonyms of valid species, the list of initial candidate species was narrowed to include only the 75 valid species. Native range, growth duration, and habit were utilized as selection criteria. Data on these characteristics were extracted from the International Legume Database and Information Service (ILDIS) (https://ildis.org/LegumeWeb/) and local floras (Krok et al., 1994; Mossberg et al., 1992; Tutin et al., 1968). Ethnobotanical data on each species were also acquired to determine if species had a history of previous human use. Occurrence data on each species was obtained by consolidating a list of herbarium specimens collected in northern Sweden from the Swedish Virtual Herbarium. The initial list of candidate species was narrowed to those native to Sweden, with a perennial growth duration and herbaceous habit, and with a minimum of 20 herbarium specimens documented.

Physical herbarium specimens from the Umeå University Herbarium (UME) for the narrowed list of candidate species were evaluated for additional characteristics of interest. The leaf width and length and plant height were measured for all specimens from the Västerbottens län collection. The location of inflorescence and phenological stage of the specimens at time of collection was also noted. A flowering period range was constructed using the date of collection for all specimens in flower. Specimen data with collection location and collector information was compiled for each measured specimen. Flowering period was used to select the final candidate species, with early flowering utilized as the main selection parameter. Descriptive statistics were calculated for the flowering day of year for each species and then for the combined dataset of all species with herbarium measurements. Species within the minimum and first quartile of the minimum or median flowering day for the dataset of all species were then selected as the final candidate species.

The Artportalen database (https://artportalen.se/) was consulted to locate populations of the final candidate species. Identified populations were first visited in June to July 2020 and large populations were selected for seed collection. Seed pod maturity was tracked for each population and mature pods were collected, dried, and threshed. Seeds were counted and weighed to calculate thousand seed weights, and subsequently stored for future cultivation (Figure 7).

Figure 7. Seeds collected from wild populations of (left to right) *Vicia sepium*, *Vicia cracca*, *Lathyrus pratensis*, *Lathyrus japonicus*, *Lathyrus palustris*, *Astragalus alpinus*, and *Anthyllis vulneraria*. Photo: Brooke Micke.

3.4 Paper IV

The collected seeds for *Lathyrus palustris*, *Lathyrus pratensis*, *Vicia cracca*, and *Vicia sepium* were sterilized in 6% sodium hypochlorite, washed with water, and allowed to air dry. Seeds were then scarified by soaking in 97% sulphuric acid, washed with water, and air-dried. Details on the sterilization and scarification soaking times can be found in **paper IV**. Dry seeds were inoculated with a *Rhizobium leguminosarum* bv. *viciae* inoculant and coated in lime at rates of 0.005 g inoculant and 0.25 g lime per 1 g of seed.

A field experiment was established at the Röbäcksdalen Field Research Station (63.81°N, 20.24°E) in 2021 with plots sown as mixed stands with the timothy cultivar Grindstad and different forage legume species. Four seed mixtures were sown in plots using a randomised complete block design composed of three blocks. All four seed mixtures contained timothy sown at a rate of 10 kg ha^{-1}. The control seed mixture also included red clover cultivar Torun at a sowing rate of 5 kg ha^{-1}. Two single legume seed mixtures included either *Lathyrus pratensis* at a sowing rate of 23.9 kg ha^{-1} or *Vicia cracca* at a sowing rate of 24.9 kg ha^{-1}. Sowing rates for the *Lathyrus pratensis* and *Vicia cracca* seed mixtures were determined according to germination rates and thousand seed weights to achieve 160 plants per m^2. A maximum biodiversity seed mixture containing all four native legume species included each species at a sowing rate to achieve 40 plants per m^2 (*Lathyrus palustris* at 4.4 kg ha^{-1}, *Lathyrus pratensis* at 6.0 kg ha^{-1}, *Vicia cracca* at 6.2 kg ha^{-1}, and *Vicia sepium* at 10.4 kg ha^{-1}). Three harvest frequencies were applied on each stand mixture; a one-, two-, and three-cut system. Each combination of stand mixture and harvest frequency was included as a single replicate per block, totaling 36 plots (Figure 8).

Figure 8. A field trial of native legume species sown in mixed stands with timothy. This image was taken following the first harvest of the three-cut system in 2022 at Röbäcksdalen Field Research Station in Umeå. Photo: Brooke Micke.

Pre-harvest data was collected from a subplot located at the center of each plot. The plant height and phenology stage of five plants of the sown legume species was documented, including five plants of all four legume species sown in the maximum biodiversity mix. The first harvest for each system was taken according to the typical harvest date for each harvest regime. The second harvest for the three-cut system was delayed due to rain and taken seven weeks after the first harvest. The second harvest of the two-cut system was also taken seven weeks after the first harvest. The final harvest of the three-cut system was taken four and half weeks after the second harvest. Plots were harvested using a mower harvester, yields were recorded, and a sample was taken for DM determination. A representative subsample was taken from the harvested material for botanical composition separation into grass, planted legume species, and broad-leaf weeds. The separated samples were then dried for DM determination. For plots sown with the control, *Lathyrus pratensis*, or *Vicia cracca* seed mixture, a sample of the planted legume species were collected and dried for nutritive value analysis. For the maximum biodiversity plots, only a subsample of *Vicia sepium* was taken, as no *Lathyrus palustris* plants established in the experiment. Plots of the *Lathyrus pratensis* and *Vicia cracca* species mixtures harvested in the third cut of the three-cut system contained insufficient amounts of the planted legume species, thus nutritive value analysis was only done on samples of red clover and *Vicia sepium*.

Nutritive value analysis for CP, aNDFom, and VOS digestibility were evaluated according to the methods presented in **paper II**. A general linear mixed model procedure in PROC GLIMMIX ((SAS software version 9.4, SAS Institute Inc., 2008) was analyzed for the output variables CP, aNDFom, VOS digestibility, DM, ash, total yield, and legume yield. Harvest number, species, and their interactions were utilized as fixed-effects factors, with block as a random-effects factor. The interaction of harvest and species was analyzed using a partitioned analysis of the mean estimates. Tukey's method ($p < 0.05$) was used to compare mean estimates for the main fixed effects and the partitioned interactions.

4. Results and discussion

4.1 Comparison of juicing and leaf stripping fractionation (paper I)

Overall, the results from this study are rather limited due to extensive complications during data collection. As this study reports data from a single production year in Norway and a single harvest in Sweden, it is not possible to draw concrete conclusions about the expected nutrient composition or yield of fractions produced by juicing and leaf stripping. This study represents an initial exploration of two fractionation methods under the specific conditions of the year of data collection and provide a first comparison of these methods when used under the same conditions.

All statements of difference are significant at the threshold of $p < 0.05$. Fractionation method had a variable impact on the CP concentration of the biorefined and residual fractions. Though the juice contained a higher CP concentration than the leaves in the first harvest from the 2020 NO dataset, there was no difference in CP content between the juice and leaves in subsequent harvests (Figure 9). This variability is driven by a difference in CP allocation between methods, as juicing was capable of allocating more of the plant's CP to the biorefined fraction in the first harvest. The phenology of plants may influence the CP allocation potential of fractionation methods. Plants in the first harvest were in an earlier phenological stage and thus would have more soluble protein and lower amounts of fiber-bound protein (Buxton, 1996). This is supported by a previous study which demonstrated that red clover plants in earlier phenological stages contain higher concentrations of true protein, the most relevant protein fraction for protein extraction by means of press juicing (Solati et al., 2017). Results from the 3rd

Cut SENO dataset reveal an inconsistency in CP allocation between sites, with the leaves containing a higher CP concentration than the juice in Sweden (Figure 3 in paper I). As data from the Swedish trial are limited, this inconsistency is challenging to explain, though could be the result of differences in use or performance of the leaf stripper and/or press juicer between locations. The functionality of fractionation machinery for both methods is heavily dependent on the human user, thus reducing the production of reproducible results. Similar trends in CP allocation were observed for the residual fractions in both datasets. Overall, the differences in CP concentration were small between the juice and leaves, and the pulp and stems, demonstrating that the impact on fractionation method on CP concentration of the biorefined and residual fractions is minor.

A clearer differentiation between methods was observed for the allocation of aNDFom to the biorefined and residual fractions. In the 2020 NO dataset, the leaf fraction had drastically higher aNDFom concentrations than the juice fraction (Figure 9). Similar results were observed in the 3rd Cut SENO dataset, where the aNDFom concentration of the leaves and juice were 338 g/kg DM and 22.6 g/kg DM, respectively (Table 5 in paper I). The difference between the mechanisms by which the two fractionation methods separate the plant can help explain their contrasting aNDFom allocation. Leaf stripping creates a biorefined fraction composed of leaves and soft, upper stems. Though this fraction contains a major proportion of the plant's soluble protein, it also includes considerable amounts of fiber from the leaves, petioles, and upper stems. Alternatively, press juicing macerates the plant and extracts a soluble protein-rich juice, excluding the plant's fiber and instead allocating it to the pulp fraction (Colas et al., 2013). Larger variation in aNDFom concentration was observed for the stem fraction compared to the pulp fraction, likely due to a larger influence of plant morphology on the aNDFom allocation of leaf stripping (Figure 3 in paper I).

Previous work on press juicing has focused mainly on the nutrient composition of the protein precipitate, making reported values for the raw products limited (Renaudeau et al., 2022; Stødkilde et al., 2020). A study in Denmark, however, focused on the nutritive value of the pulp fraction, reporting CP concentrations of 198 g/kg DM in pulp produced from plants with a CP of 203 g/kg DM (Damborg et al., 2018). This is higher than the CP concentrations for pulp fractions produced in Norway in this study, though the difference can be attributed to lower CP concentrations in the original plant (Table 1 in paper I). As for fiber concentration of the pulp fraction, results from the Danish study support the high fiber content observed in the pulp produced in both locations of this study (Damborg et al., 2018). Studies on the leaf stripping of red clover are also few, as the majority of work has focused on lucerne (Andrzejewska et al., 2020; Shinners et al., 2007). Two previous studies on red clover have reported CP concentrations of the leaf stripped fraction between 262-268 g/kg DM (Liebhardt et al., 2022; Pleger et al., 2021). Larger variability in CP concentration was observed in this study, however, with values from 200-241 g/kg DM in the 2020 NO dataset and from 247-316 g/kg DM in the 3rd Cut SENO dataset. Leaf stripper fraction CP concentrations are heavily influenced by the CP concentrations of the whole plant, explaining a large proportion of the variability observed. The Pleger study also reported on the crude fiber concentration of the leaf stripped fraction, though the values were considerably lower than the aNDFom values observed in this study. This disparity can be partially attributed to the difference in analysis method, as the two methods do not equally represent the hemicellulose and lignin present (Möller, 2014). Overall, the differences in nutrient composition observed between locations within this study and between this study and previous studies are likely heavily influenced by machine settings and functionality. Standardization of leaf stripper machinery and machine settings based on stand characteristics will be an essential aspect in assuring consistency in the nutrient composition of the resulting fractions.

The influence of fractionation method on yields of the biorefined and residual fractions was variable (Figure 9). Juice fractions produced higher yields than leaf fractions in the first and third harvests in Norway. No difference in yield, however, was observed in the second harvest in Norway or the third harvest in Sweden (Figure 3 in paper I). The higher juice yields in Norway can potentially be explained by the higher proportion of biomass allocated to the biorefined fraction for juicing compared to leaf stripping (Supplementary material Figure 2 in paper I). Alternatively, the allocation of biomass in Sweden was consistent between fractionation methods (Supplementary material Figure 4 in paper I). The lower yield allocation to the leaf fraction in Norway is potentially the result of a lower proportion of legume in the stand, thus providing less leaf biomass for leaf stripping (Table 1 in paper 1). Additional work is required to determine how the botanical composition of the stands impacts yields of fractions produced from leaf stripping. Yields of the residual fractions were more consistent between fractionation methods, with the only difference between the pulp and stems seen in the first harvest in Norway. Overall, higher variability in yield allocation was observed for leaf stripping compared to juicing. Previous studies have reported results on the biomass allocation of both fractionation methods in pure stands of red clover. Liebhardt et al. (2022) saw higher yield allocations to the leaf fraction than observed in this study. The differences in biomass allocation could be the result of differences in plant morphology, phenological stage, stand density, leaf stripper functionality, or a combination of all these factors, as leaf stripping seems to be heavily influenced by stand characteristics. Alternatively, higher allocation of biomass to the juice was seen in this study compared to previous studies (Damborg et al., 2020; Santamaría-Fernández et al., 2017). This disparity is challenging to explain, though in the case of the Damborg study is likely partially due to the inclusion of loss proportion in the mass balance calculation.

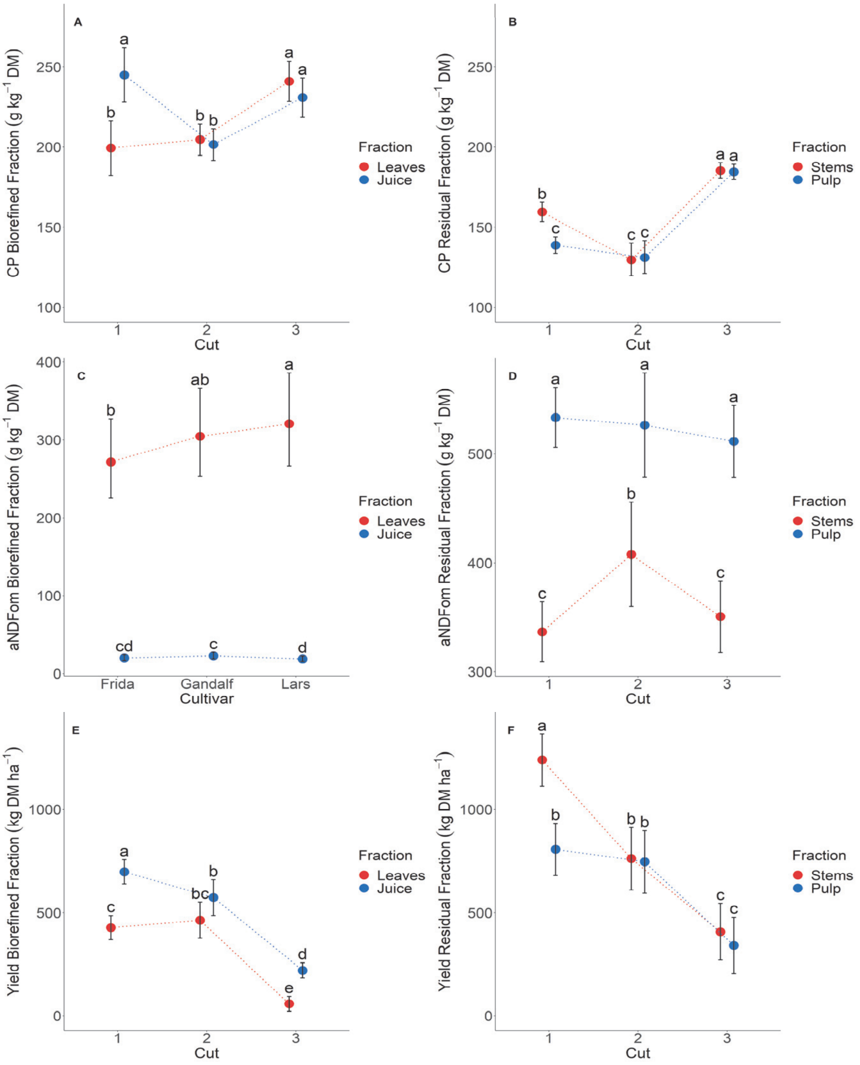

Figure 9. Least square means from the 2020 NO dataset of (A) CP concentration of the biorefined fraction in response to the interaction of cut and fractionation method; (B) CP concentration of the residual fraction in response to the interaction of cut and fractionation method; (C) aNDFom concentration of the biorefined fraction in response to the interaction of cultivar and fractionation method; (D) aNDFom concentration of the residual fraction in response to the interaction of cut and fractionation method; (E) Total yield of the biorefined fraction in response to the interaction of cut and fractionation method; (F) Total yield of the residual fraction in response to the interaction of cut and fractionation method. These graphs are only for significant interactions. Vertical bars represent 95 % confidence intervals. Means with common letters are not different ($p >$ 0.05) according to Tukey's test.

4.2 Potential of leaf stripping mixed stands (paper II)

This study demonstrated that the leaf stripper is capable of fractionating mixed stands, harvesting roughly a third of the total biomass and upwards of 50% of the clover biomass. The success of leaf stripping mixed stands is likely heavily dependent on the morphology and phenology of the clover component, as well as the machine settings. Adjustment of the machine settings should be a continuous process, with speed and rotor settings being altered dependent on the visual assessment of the LSF and RF.

The pre-harvest (CF, GF, sward) and post-harvest (LSF and RF) fractions varied in terms of nutritive value (Figure 10). All statements of difference are significant at the threshold of $p < 0.05$. In both years, the LSF had a higher CP concentration than all other fractions, increasing the CP concentration by 57.4 g/kg DM in 2021 and 73.6 g/kg DM in 2022 compared to the sward. These results were consistent across both years, with no difference in CP concentration of the same fraction between years. The LSF had the lowest aNDFom concentration in 2021 and was lower than all other fractions apart from the CF in 2022. Leaf stripping successfully produced a LSF fraction with a lower aNDFom content compared to the sward, with the aNDFom concentration decreasing by 110 g/kg DM and 70.5 g/kg DM in 2021 and 2022, respectively. The aNDFom concentration of the LSF was higher in 2022 than in 2021, likely due to the higher proportion of grass in the sward in 2022. Visual assessment of the LSF revealed it was composed of both clover leaves and grass. A higher grass proportion of the sward would likely result in a larger grass contribution to the LSF, thus increasing its aNDFom content. Hand separation could elucidate the contribution of the grass fraction to the LSF and provide additional insight into its nutritive value. A previous study on leaf stripping pure stands of red clover determined that 82% of the LSF was composed of clover leaves, though additional work is needed to determine the composition of the LSF produced from mixed stands (Liebhardt et al., 2022). The LSF had higher digestibility than all other fractions, increasing the digestibility of the sward by 3.25%. Overall, leaf stripping resulted in a LSF with improved nutritive value. The primary effect of leaf stripping was an increase in CP concentration, with average increase of 40%. Decreased aNDFom content and improved digestibility were secondary effects, due to the variable decrease in aNDFom concentration (27.3% in 2021, 16.0% in 2022) and modest increase in digestibility.

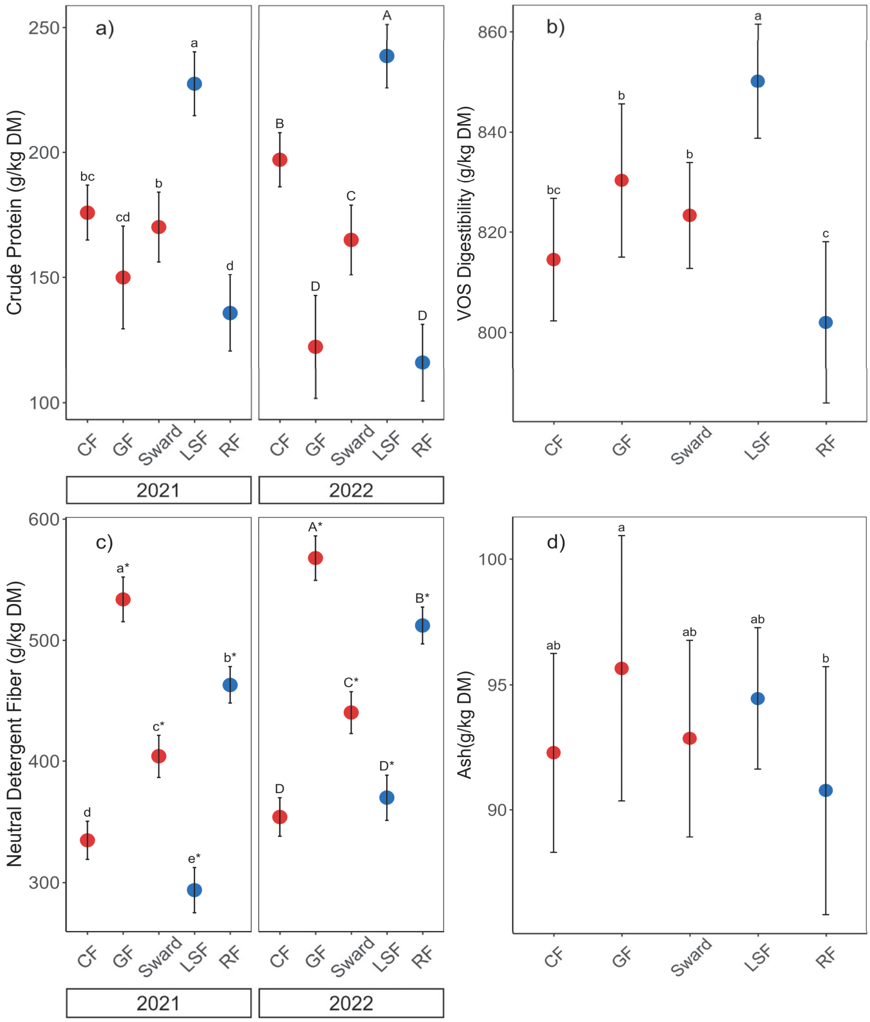

Figure 10. Crude protein (a), VOS digestibility (b), neutral detergent fiber (c), and ash (d) of different plant fractions, pre-harvest (red) and post-harvest (blue) using a leaf stripper (LS) in mixed grass-clover leys at Röbäcksdalen Field Research Station in 2021 and 2022. Points are least squares means (n=20 for (a) and (c) and n=40 for (b) and (d)). Error bars are 95% confidence intervals. Means with common letters are not different ($p < 0.05$) according to the Holm-Bonferroni method for subfigures (a) and (c) or Tukey's test for subfigures (b) and (d). Sub-figures (a) and (c) present nutritive value data for the interaction of year and fraction. Same fractions across years 2021 and 2022 that are statistically different are denoted with an asterisk. The clover fraction (CF) and grass fraction (GF) constitute the sward pre-harvest. The post-harvest fractions include the leaf stripper fraction (LSF) and residual fraction (RF).

The regression models constructed using the dataset with all explanatory variables outperformed the models utilizing the dataset limited to explanatory variables with moderate or strong correlation to the response variables. Overall, the constructed models performed quite poorly and are likely not an effective method for predicting the nutritive value of the LSF. The full model for all four response variables had adjusted R^2 values ranging from 0.64-0.69, however, were heavily reliant on the nutritive value parameters of the pre-harvest fractions. Alternatively, the field models explained far less variability, with adjusted R^2 values between 0.25 and 0.49 (Table 3 in paper II). As data on the nutritive value of the sward is not available prior to harvest, the field models best represent the pre-harvest prediction potential. The addition of field spectrometers could provide an opportunity to estimate the nutritive value of the sward before harvest, however the financial investment required would limit the scale of their use (Morel et al., 2022).

4.3 Utility of fractions and integration of forage fractionation

The fractions produced from juicing and leaf stripping are only relevant if they can increase the feed value of grassland forage for monogastrics and be produced on an industrial scale. Though the results of **paper I** demonstrate that juicing is capable of producing a biorefined fraction with a high CP content and consistently low aNDFom concentration, the multi-step processing required presents challenges for its integration. Time constraints between harvest and fractionation exist for press juicing, thus requiring the fast collection, transportation, and processing of forage (Jørgensen et al., 2022). The largest hurdle to overcome, however, is the establishment of localized fractionation facilities capable of large-scale juicing and protein precipitation. These facilities would require both specialized machinery and labor, resulting in high costs for production. The economic viability of industrial scale press juice fractionation will heavily depend on the breadth of applications for the produced fractions (Kromus et al., 2004). Previous studies have demonstrated that protein precipitate produced from the juice fraction is a viable protein feed for monogastrics (Renaudeau et al., 2022; Stødkilde et al., 2020). The high feed value of the protein precipitate could provide a locally produced alternative to imported soybean products, thus offsetting the financial investment required for its production and simultaneously improving the sustainability of monogastric livestock production. Additionally, the pulp fraction has potential as a ruminant feed due to its comparable protein concentration to the original plant, allowing for the utilization of all products from fractionation process (Damborg et al., 2018). The integration of press juice fractionation in the Nordic region would require the successful production of protein precipitate with high feed value from mixed grass-legume swards. Though the results of this study solely focus on the potential of juice fractions from pure legume stands, a previous study demonstrated that protein precipitate produced from grass-clover mixtures provided a valuable feed for poultry production (Stødkilde et al., 2020).

The feed value of fractions produced by leaf stripping and the integration potential of the method are far less understood, however. Leaf stripping fractionation methods are underdeveloped and studies have yet to determine standardized practices for its use. Previous studies have utilized a variety of leaf stripping machinery, thus preventing uniform data collection to evaluate the potential of the method. The results from **paper I** demonstrate that the leaf fraction contains similar CP concentrations to juice; however, the high aNDFom concentrations may pose problems for its value as a protein feed for monogastrics. The further investigation of the nutritive value of leaf stripper fractions produced from mixed stands in **paper II** further demonstrated that though leaf fraction has a lower aNDFom concentration than the sward, its relatively high aNDFom concentration may still be too high to serve as a protein feed for monogastrics. A feeding trial investigating the nutritive value of protein pastes and ensiled leaves of forage legumes for pigs concluded that ensiled leaf fractions should not be considered as a viable protein feed, but rather an energy feed source due to their high fiber content and its negative impact on digestibility (Renaudeau et al., 2022). Further processing of the leaf fraction is likely necessary to achieve a nutritive value suitable for monogastrics. The cost of machinery and development of appropriate methods of preservation also pose challenges to the integration of leaf stripping. As current leaf stripping methods are unable to produce fractions suitable for replacing soybean in the diet of monogastrics, the increased cost of purchasing leaf stripping machinery is likely not worth the investment. Additionally, current preservation methods, such as ensiling leaves with grain or additives to achieve appropriate DM, result in protein degradation, further decreasing the nutritive value of the leaf fraction (Renaudeau et al., 2022; Shinners et al., 2007). Though leaf fractions are not a suitable protein feed for monogastrics, they could be used to increase the digestibility and CP content in the feed rations of lactating dairy cows, though the additional protein may not be fully utilized for productive purposes (Vanhatalo et al., 2009). The stem fractions could serve as a feed source for ruminants with low energy requirements, such as dry dairy cows or heifers. Considerable work is still required to determine the potential of leaf stripper fractionation and evaluate whether the produced fractions are suitable feed sources for monogastrics and ruminants.

4.4 Selection of native legume species (paper III)

Based on the parameters set for the initial selection of the 75 wild legume species, 17 species were identified for further exploration by means of herbarium survey. Species included in the herbarium survey were *Anthyllis vulneraria* L. subsp. *lapponica* (Hyl.) Jalas, *Astragalus alpinus* L. subsp. *alpinus*, *Astragalus frigidus* (L.) A. Gray, *Astragalus norvegicus* Weber, *Lathyrus japonicus* Willd. subsp. *maritimus* (L.) P.W. Ball, *Lathyrus palustris* L., *Lathyrus pratensis* L., *Medicago lupulina* L., *Melilotus albus* Medik., *Oxytropis lapponica* (Wahlenb.) Gay, *Trifolium aureum* Pollich, *Trifolium medium* L., *Trifolium spadiceum* L., *Vicia cracca* L., *Vicia hirsuta* (L.) Gray, *Vicia sativa* L. subsp. *sativa*, and *Vicia sepium* L. subsp. *montana* (W.D.J. Koch) Hämet-Ahti. Of these 17 species, 13 had previous record of ethnobotanical use as a forage. A species' historical record of utility as forage can highlight its agronomic potential for integration in ley systems. The ethnobotanical history of wild species present an opportunity to focus research efforts on species that have previously demonstrated potential for cultivation. The importance of ethnobotanical records has been highlighted by other researchers, particularly when evaluating the potential of wild species for de novo domestication (Ciotir et al., 2019; Frawley et al., 2020; Leakey, 2019).

The specimens measured in the herbarium survey had been collected throughout the entire province of Västerbotten, thus representing the phenological and morphological diversity of populations throughout the region. Results from data on the flowering date range of the surveyed species identified seven species that were within the identified parameters for early flowering (Figure 11). The final candidate species were *Anthyllis vulneraria*, *Astragalus alpinus*, *Lathyrus japonicus*, *Lathyrus palustris*, *Lathyrus pratensis*, *Vicia cracca*, and *Vicia sepium*.

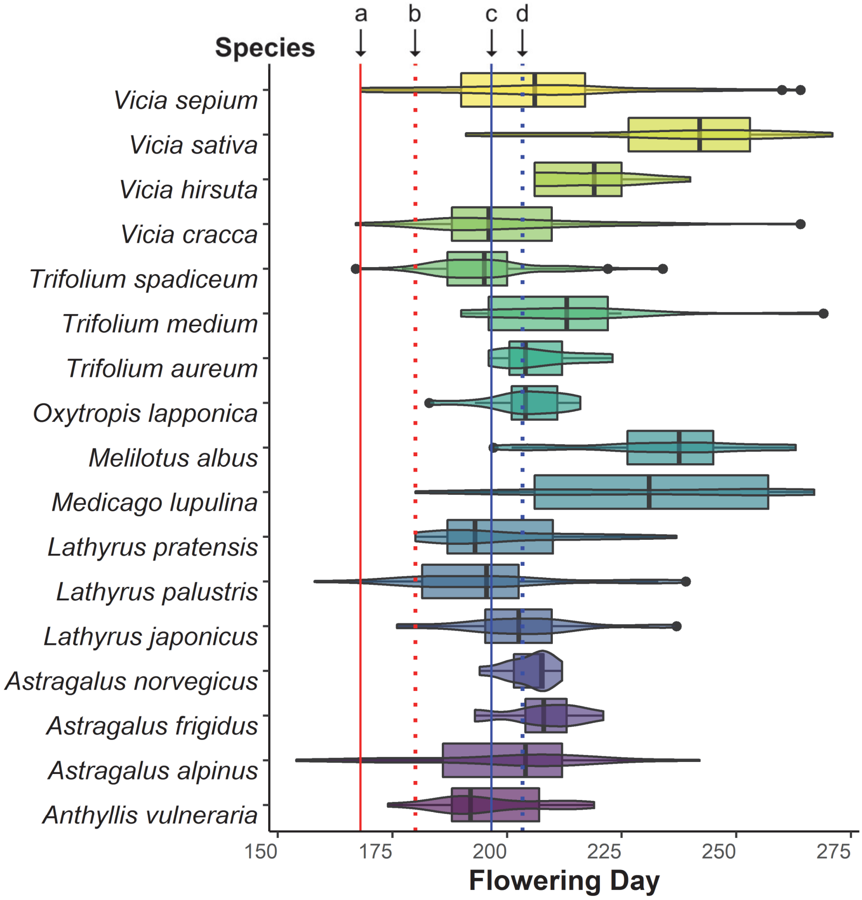

Figure 11. Flowering day of the herbarium specimens measured. The day of flowering is expressed as x of 365, to represent the day of the year out of the total 365 days. Line a) represents the first quartile of the earliest flowering day, b) represents the median of the earliest flowering day, c) represents the first quartile of the median flowering day, and d) represents the median of the median flowering day. Left edge of rectangular plot represents 1st quartile, black vertical lines represent median, and right edge represents 3rd quartile. Black points represent outliers.

Flowering date was chosen as the major criterion for selection, as the provisioning of floral resources is an important ecosystem service provided by grassland agricultural systems. Early flowering legume species have the potential to contribute an abundance of resources to local pollinators at the start of the season when leys are void of floral resources (Johansen et al., 2019). Agriculturally relevant legume species represent a major proportion of the floral resources utilized by pollinators, particularly in agricultural landscapes which often lack floral diversity (Decourtye et al., 2010; Kleijn & Raemakers, 2008; Lagerlöf et al., 1992). By increasing the legume biodiversity through the inclusion of native species, leys can become more sustainable and contribute an essential ecosystem service to local pollinator populations. Additional morphological traits were measured during the herbarium survey, however were not identified as selection criteria due to potential biases in specimen data. Plant height was not included in selection, as the plant heights recorded from the specimens were considerably lower than the heights recorded in local floras (Tutin et al., 1968). The plant height of forage legumes is an important aspect of their agronomic potential, as short plants would have difficulty competing with grasses for light. However, the bias seen in the herbarium survey inhibited the accurate collection of plant height data. Biases in herbarium data are well recorded and are often the result of particular interest of the collector or adjustments made to ease collection (Daru et al., 2018; Moerman & Estabrook, 2006). These biases should not deter researchers from utilizing herbarium specimens for data collection on plant diversity; however, it is essential that bias potential be acknowledged when reporting results.

In addition to potential biases in herbarium specimens, data accuracy from botanical databases must also be considered. Habit data extracted from ILDIS listed several of the species from the initial candidate list as perennial. Data extracted from other databases contradicted this, listing the species as annual or biennial (Ciotir et al., 2016; POWO, 2022; Roskov et al., 2005; Tutin et al., 1968). Following discussions with local experts, it was determined that the species in question did not have perennial habits in northern Sweden. One of these species, *Trifolium spadiceum*, was initially selected as a final candidate species, but was subsequently removed from the list and replaced by *Lathyrus japonicus* (Figure 11). The immense amount of data compiled in botanical databases, presents difficulties for the validation of recorded data. Due to the time-consuming nature of data quality assessment, validation and verification of biodiversity data is often overlooked (Dalcin et al., 2012). Regardless, data quality practices are essential to assuring the accurate utilization of biodiversity databases and should be a prioritized aspect of database infrastructure (Chapman, 2005). Researchers extracting data from these resources must take the necessary steps to cross-reference the data and consult experts as an additional verification step prior to the reporting of results.

In total 44 populations of the seven final candidate species were visited in Västerbotten, of which 26 were selected for seed collection based on their proximity to Umeå and sufficient population size. Additional populations were identified in Artportalen, but during the initial visit to the site, no plants of the intended species were found. The discrepancy could be attributed to population decline or to incorrect spatial data. Seeds were collected from the selected sites from August to October in 2020, with seed maturity characterized by pods with a brown or black color and nearing dehiscence. *Anthyllis vulneraria* had the shortest collection time (11 days), while *Vicia sepium* had the longest (61 days). Thousand seed weights were variable between species, ranging from *Astragalus alpinus* with a mean of 1.12 g and standard deviation 0.13 g to *Lathyrus japonicus* with a mean of 35.2 g and standard deviation of 2.63 g. The collection of seed from wild species with agronomic potential represents a major step in connecting the recommendation of wild species to their eventual integration. Identification of crop wild relatives with potential for de novo domestication often stops at their recommendation (Ciotir et al., 2019; Frawley et al., 2020). By collecting seed for cultivation and additional study of the agronomic potential of the identified species, this study demonstrates clear methods to bridge the gap between selection and integration.

4.5 Agronomic potential of selected native legumes (paper IV)

The four sown legume species from the various seed mixtures were harvested in varying phenological stages and plant heights. *Trifolium pratense* and *Vicia cracca* had the largest variability in phenological stage between harvests, with all four phenological stages occurring in both species. Alternatively, *Lathyrus pratensis* was harvested in the vegetative stage for all harvests apart from the one-cut system. Little variability in phenology arose for *Vicia sepium*, with all plants in the vegetative or bud stage during harvest. All species were in the latest stage of maturity and had the tallest plant height in the one-cut system. As these native legume species were selected in **paper III** based on their ability to provide pollinator resources early in the season, the phenological stage at harvest is an important factor to consider. Based on the results from the first production year, *Vicia cracca* advances to the flowering stage earlier than the other studied species. *Lathyrus pratensis* and *Vicia sepium* plants did not flower in any harvest apart from the one-cut system, demonstrating slower phenological development than what is observed in wild populations. This delayed development could be due to the young age of the stand and alternative flowering times could occur in later years of production.

All statements of difference are significant at the threshold of $p < 0.05$. The native legume species had higher CP concentrations than *Trifolium pratense* (Figure 1 in paper IV). The low CP values observed in this study for *Trifolium pratense* (170 g/kg DM) are considerably lower than the reported CP concentrations from variety trials of the same cultivar in the north using a three-cut system (232-275 g/kg DM) (Sandström & Barrlund, 2021). As the CP concentration from this study represents the combined data across all harvests due to no significant interaction, the low CP concentration is likely influenced by the lower quality of plants harvested in the one-cut system. This is supported by the results for CP concentration of the different harvests, in which each of the harvests had different CP concentrations, with the highest CP occurring in the first harvest of the three-cut system (243 g/kg DM) and the lowest in the sole harvest of the one-cut system (160 g/kg DM). The variable CP concentration between harvests is likely the result of maturity stage, as forages harvested at earlier maturity stages contain higher concentrations of CP (Buxton, 1996; Elgersma & Søegaard, 2018). The high CP concentrations of *Lathyrus pratensis* (213 g/kg DM), *Vicia cracca* (229 g/kg DM), and *Vicia sepium* (212 g/kg DM) show promise for their ability to contribute protein to the sward in later years of production.

There was a distinct delineation between *Lathyrus pratensis* and *Vicia cracca*, and *Vicia sepium* and *Trifolium pratense* in terms of aNDFom concentration, with *Lathyrus pratensis* and *Vicia cracca* having a higher aNDFom in nearly all harvests (Figure 12A). The high aNDFom concentrations in *Lathyrus pratensis* are likely the result of the large proportion of thick cell walls reported for the species (Zoric et al., 2011). *Vicia cracca* had consistently high aNDFom concentrations across all cuts, demonstrating a minimal impact of maturity stage on the species' aNDFom content (Figure 12B). The aNDFom concentrations observed in this study are comparable with values reported in other studies (Ciftci et al., 2021; Gürsoy, 2021). *Vicia sepium*, however, had low aNDFom concentrations in each harvest, further demonstrating its promising nutritive value.

Lathyrus pratensis had poor digestibility regardless of harvest frequency, which was lower than all other legume species across all harvests (Figure 12C & D). This low digestibility is likely related to its high aNDFom content and the large proportion of cell walls present in the species (Zoric et al., 2011). In contrast, *Vicia cracca* had consistently high digestibility in all harvests. Considering the similarity in aNDFom concentration between the two species, further explanation is needed to understand their disparate digestibility. The poor digestibility of *Lathyrus pratensis* could be due to a higher proportion of lignin in its fiber concentration compared to *Vicia cracca* (Moore & Jung, 2001). Another explanation could be the presence of anti-nutritional factors, such as condensed tannins, which have been reported for other species in the genus (Bate-Smith, 1973). Promising digestibility results have been previously reported for *Vicia cracca*, with the species having comparable digestibility to *Medicago sativa* (Gürsoy, 2021). Considering the similarity in digestibility between *Vicia cracca* and *Trifolium pratense* in this study, the species demonstrates similar digestibility to the two most important forage legume species in the world. Analogous to the results for CP and aNDFom, the digestibility of *Vicia sepium* was comparable or superior to that of *Trifolium pratense* in all harvests. The consistently high nutritive value of *Vicia sepium* indicates a promising feed value for ruminants and warrants further investigation into its inclusion in mixed swards.

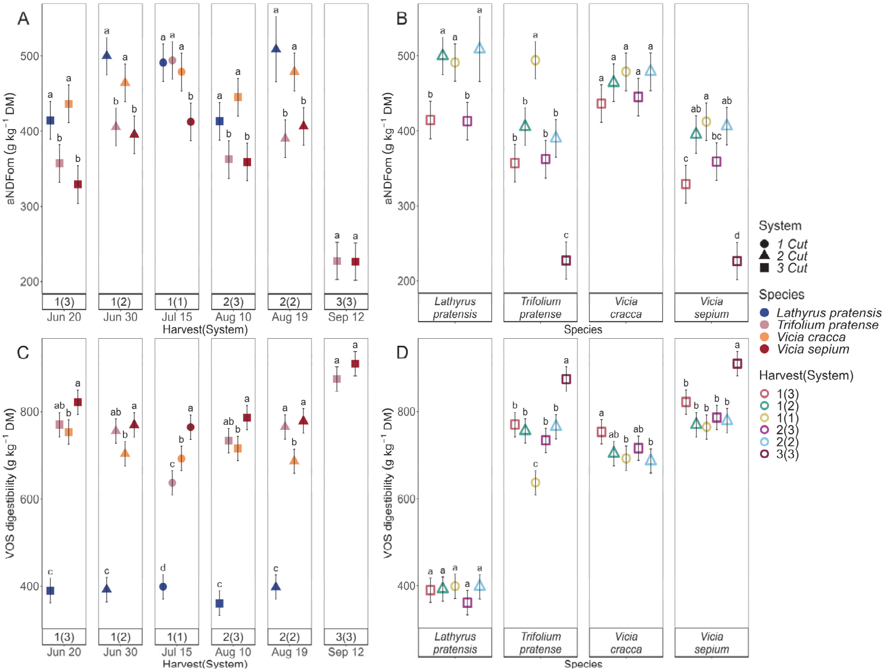

Figure 12. Least square means of (A) neutral detergent fiber (aNDFom) concentration in response to the interaction of harvest and species sliced by harvest number; (B) aNDFom concentration in response to the interaction of species and harvest sliced by species; (C) rumen degradable organic matter (VOS) digestibility in response to the interaction of harvest and species sliced by harvest; (D) VOS digestibility in response to the interaction of species and harvest number sliced by species. Graphs A and B present the same least squares means sliced with different fixed effects. Graphs C and D present the same least squares means sliced with different fixed effects. These graphs are only for significant interactions. Vertical bars represent 95 % confidence intervals. Means with common letters are not different ($p > 0.05$) according to Tukey's test.

Results for total yield were not highly informative as species mixture had little effect on the yield of the mixed sward, with no difference between species mixtures in any harvest apart from the one-cut system, where total yield of the *Trifolium pratense* mix was higher than all native legume species mixtures (Figure 3A & B in paper IV). This is unsurprising, as lower legume density in mixed stands is often supplemented by an increase in grass biomass. Legume yields provided more information on differences in biomass production between the legume species. For the all harvests, legume yields of *Lathyrus pratensis* were the lowest (Figure 3C & D in paper IV). Based on the low yields of *Lathyrus pratensis*, the species has poor productivity when grown in mixed stands in the first year of production.

Legume yields of *Vicia cracca* and the biodiverse mix were comparable across all harvests. Overall, *Trifolium pratense* had the highest legume yield in each harvest, though its yields were not higher than *Vicia cracca* or the biodiverse mix in three out of the six harvests. The consistency in legume yield between *Vicia cracca* and the biodiverse mix suggests that higher levels of biodiversity have little impact on legume yield, as least in the first production year. Apart from this study, no data exists on the yield potential of these three native legume species in the Nordic context. Yield data in the subsequent production years will provide important information on the biomass potential of these species.

4.6 Expanding the knowledge on wild species in agriculture

Papers III and **IV** have demonstrated the potential for the selection of wild species with agronomic potential by linking the selection process of wild species with the evaluation of their nutritive value and yield characteristics. The 75 legume species with distribution in northern Sweden were narrowed to focus on four species, which demonstrated promise for cultivation and utility as forage. *Lathyrus pratensis*, *Vicia cracca*, and *Vicia sepium* established well in an agricultural context and showed potential for inclusion in ley systems, thus supporting the use of botanical resources to highlight wild species with agricultural promise, as demonstrated in **paper III**. Expanding the knowledge on key agronomic traits of wild species is necessary to support the evaluation of new species for domestication. Ciotir et al. (2019) noted that the acquisition of data on agriculturally relevant traits of wild species presented a major challenge in the construction of Perennial Agriculture Project Global Inventory. Improved collaboration between botanists and agronomists can facilitate increased compilation of relevant data on wild species of agricultural interest.

Ecotypes, “distinct genotypes (or populations) within a species, resulting from adaptation to local environmental conditions”, may present a less time-consuming alternative to large-scale domestication to increase agrobiodiversity in local agriculture systems (Hufford & Mazer, 2003). The utilization of ecotypes in agriculture can act to both improve crop adaptability to the local environment and conserve the genetic diversity found in local ecotypes. The production of local ecotype “cultivars” would require some degree of plant breeding on a local scale, as selected ecotypes would be most suitable in their region of origin. Development of ecotype "cultivars" could entail minor selection or even solely multiplication for seed production purposes. The cultivation of local ecotypes could be combined with need for conservation and identification of agriculturally relevant wild species. Local ecotypes could be identified and integrated into agricultural systems to both increase agrobiodiversity and conserve gene pools with the potential for future crop improvement.

A variety of characteristics must be evaluated prior to the formal integration of wild species into agricultural systems, such as leys. The results of **paper IV** provide key information on the nutritive value and yield of the selected wild legume species; however, additional traits still require assessment. The evaluation of persistence, nitrogen fixation potential, rhizobia specificity, presence of anti-nutritional factors, and seed production viability is still an essential aspect of determining their agronomic potential. Based on the results of the first year of data collection from the field trial, *Vicia sepium* demonstrates encouraging nutritive value, though additional data collection is necessary to determine if its high nutritive value is maintained in later years of production. Despite the high aNDFom concentration observed in *Vicia cracca*, its promising CP content, digestibility, and yield warrant additional evaluation of its agronomic potential. Both species are capable of spreading vegetatively through rhizomes and thus could increase their plant density following the decline of red clover to maintain a stable legume component in the stand. The results from **paper IV** only represent a single production year and additional years of data collection are underway to evaluate the nutritive value, yield, and persistence of these native legume species in subsequent years of production. Additional data collection can also help elucidate the influence of harvest frequency on their agronomic characteristics.

4.7 Implications of the diversification of legume species and forage utility

The results of this thesis demonstrate the potential of diversification both in the production and utilization of forage legume crops. By diversifying the crops grown and the breadth of their utility, challenges at both ends of the production system can be addressed. The work in this thesis focuses on production in a localized context, highlighting key issues in forage production in the Nordic region. By applying the studied methods to a specific geographic area, this thesis is able to assess the potential of new species and fractionation methods when utilized within the typical production parameters of the region. This local assessment can help determine the optimal methods for integration, as it considers local management strategies to develop solutions best suited to the needs of farmers in the region.

The diversification of forage utility functions to address the challenge of dependency on soybean as a monogastric feed. As highlighted previously in this thesis, the current reliance on soybean diminishes the sustainability of monogastric livestock production. Fractionation of forages presents an opportunity to locally produce monogastric feed from green biomass that is readily available in the Nordic countries. The results from **paper I** demonstrate the potential of press juicing to produce suitable protein feed and provide new information on the differences in nutrient and yield allocation between press juicing and leaf stripping. Though the results from **papers I** and **II** indicate excessive fiber content in leaf stripped fractions for monogastric suitability, leaf stripping may still be a useful method to improve the nutritive value of forage. Further work is needed to optimize the method and assess possible uses for the resulting fractions.

There is a consensus among many scientists, that crop diversification could provide solutions to many of the current and future challenges facing our food production systems. Increased crop diversity can serve to improve biotic and abiotic stress adaptation, decrease reliance on inputs, and provide a host of ecosystem services (Krug et al., 2023). The native legume species investigated in this thesis can assist in addressing key challenges facing forage production in the region, such as increasing persistence, decreasing the dependence on nitrogen fertilizer, and improving the sustainability of the system through ecosystem services in the form of diverse pollinator resources. Agricultural biodiversity can exist in many systems, utilizing a variety of plant taxa to provide specific contributions to the resilience and sustainability of agricultural production. The methods utilized in **paper III** are not limited solely to the investigation of wild forage legumes for leys, but can be expanded to cover a variety of agricultural systems and plant groups. Selection of wild species with agronomic potential must be followed up with the evaluation of their nutritive value and biomass productivity to increase the likelihood of their eventual integration, as demonstrated in **paper IV**.

5. Conclusions

The studies included in this thesis addressed two aspects of the diversification of forage production in the Nordic context; the diversification of forage utility to extend its feed value to monogastrics and the diversification of forage legume species cultivated to include wild, native species. Based on the results of these studies, the following conclusions can be drawn:

- Juicing produced a biorefined fraction with greater suitability as a protein feed for monogastrics due to its consistently lower aNDFom concentration than the biorefined fraction produced by leaf stripping.
- Stand characteristics, such as phenology, height, and botanical composition, influence the crude protein concentration and yield of fractions produced by leaf stripping, resulting in variability in the fractions produced.
- The machine functionality of both fractionation methods was influenced by the human user and resulted in variability in the nutrient composition and yield of the resulting fractions.
- Leaf stripping showed promise in its ability to fractionate mixed stands and produced a biorefined fraction with higher nutritive value than the sward, though a high aNDFom concentration prohibits its utility as a protein feed for monogastrics.
- Botanical resources, such as herbaria, floras, and databases, provided a time efficient survey method to assess wild species for key traits of interest. Local floras and herbarium specimens are an essential component of assessment, as they provide morphological and phenological data in a regional context and

allow for a better understanding of how wild species may perform in local agricultural systems.

- *Lathyrus pratensis*, *Vicia cracca*, and *Vicia sepium* established well in mixed stands, demonstrating their potential for cultivation.
- *Vicia cracca* was in flower during the harvests of the one- and two-cut system, demonstrating its potential to provide pollinator resources in the first production year in leys harvested only once or twice over a season.
- *Vicia sepium* and *Vicia cracca* have promising nutritive value under one-, two-, and three-cut harvesting frequencies in the first year of production.
- The consistently poor digestibility of *Lathyrus pratensis* may inhibit its inclusion in ley systems, particularly if it were to comprise high proportions of the stand composition in later years of production.

6. Future perspectives

Due to the challenges encountered in the field trials utilized for **paper I** of this thesis, additional data collection comparing the two fractionation methods is required to better understand the nutrient and yield allocation of each method under equivalent conditions. Considering the abundance of work already done to investigate the production, utility, and integration of fractionation by press juicing and the superior nutrient composition of the resulting biorefined fraction, press juicing appears to be the most probable fractionation method to achieve large-scale incorporation into agricultural production systems in the near future. However, the large investment needed to establish the necessary facilities will be a major hurdle in its integration. Considerable work is still needed to determine the potential of leaf stripping, as the results presented in this thesis and previous studies demonstrate variability in the nutritive value of the leaf and stem fractions. Hand sorting of the resulting leaf stripped fractions from both pure legume and mixed stands could potentially explain some of the variability that occurs due to differences in stand composition and leaf stripper functionality, thus enabling adjustment and optimization of the method. Additionally, studies on the implication of altering machine settings on the composition and nutritive value of the leaf stripper fraction may elucidate optimal settings for increasing forage quality, though this may result in decreased yields of the leaf stripper fraction. Further processing of leaf fractions through press juicing could be explored to determine if additional processing could produce a biorefined fraction with nutritive value high enough to justify the increased processing costs. Additional work is required to determine the utility of fractions produced by leaf stripping, making feeding trials an important step in understanding the method's potential. Previous work demonstrated that the leaf fraction is likely not suitable as a protein feed for monogastrics (Renaudeau et al., 2022). However, feeding trials with a focus on highly productive dairy cows could be of interest to determine if the additional protein and higher digestibility of the leaf fraction can increase productivity. Additionally, feeding trials utilizing stem fractions as a forage source for less productive ruminants, such as dry cows and heifers, could provide useful information on the potential of the method's residual fraction. Commercial leaf stripping machinery is not currently available, thus advancement in machine development is a necessary aspect of the large-scale integration of the fractionation method.

The selection process demonstrated in **paper III** could be applied to other regions in Sweden and the Nordics to determine if additional wild species could be suitable for inclusion in leys. As *Vicia cracca* and *V. sepium* have a native distribution throughout the majority of the Nordic region, their potential for inclusion spans beyond just leys in northern Sweden (Mossberg & Stenberg, 2018). Additionally, alternative traits of interest could be utilized to select additional species in northern Sweden based on other biodiversity goals. Seed collection and subsequent production could be broadly applied to the Nordic countries or regionally focused to maintain high genetic diversity and ensure adaptation to the local environment through the utilization of local ecotypes. As for the species studied in this thesis, further assessment of their nutritive value and biomass potential is underway with an additional year of data collection in 2023. Continued study of this field trial in the following years will provide useful data on the persistence and shift in stand composition following the decline of red clover. The work in this thesis represents a promising start, though data is still lacking on aspects such as nitrogen fixation capacity, rhizobia specificity, and pollinator resource availability. Outside of this thesis, a study on the presence of condensed tannins, phytoestrogens, and saponins in the seven species selected in **paper III** is in progress. Identifying any potential anti-nutritional factors and determining their concentrations will be essential to assuring the inclusion of these wild legume species will not have negative impacts on the health of livestock. Perhaps the most important aspect of future studies on these wild species will be their potential for seed production on a scale large enough to guarantee economic viability. Various stakeholders, such as agronomists, farmers, and seed companies, should be involved in this process to develop seed mixtures suited to the needs of ley production systems.

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Popular science summary

Forage, grasses and legumes grown to feed livestock, are an important part of the agricultural sector in the Nordic countries. Due to the long daylight hours and mild temperatures, the climate in this region is ideal for the production of forage with an optimal nutrient composition. Forages in this region are typically grown in mixed leys, in which grasses and legumes are grown as part of a crop rotation. Leys represent upwards of 75% of the arable land in northern Sweden and supply the feed necessary for dairy production. This thesis explores methods to diversify forage production both in terms of how it can be used and which species of forage legumes are grown.

Diversifying the use of forage is of particular interest in the production of monogastric animals, such as pigs and chickens. Monogastrics require feed with a high protein concentration and minimal fiber, due to a much lower capacity to digest unprocessed forage fibers compared to ruminants. Currently, large quantities of soybean are imported to feed monogastrics. The production and import of soybean is not environmentally sustainable, thus the local production of monogastric feed could assist in improving the sustainability of the system. Previous studies have explored the potential of fractionation of forages to create a feed product with suitable nutrient composition for monogastrics. This thesis focuses on two methods of fractionation, press juicing and leaf stripping. Press juicing works by macerating the forage and separating it into two fraction, a protein-rich juice and a fiber-rich pulp. Leaf stripping, on the other hand, creates fractions by separating the protein-rich leaves from the fiber-rich stems.

Diversification of the species grown in leys can help address issues encountered during forage production and provide ecosystem services to increase its sustainability. Leys in northern Sweden typically include red clover as the main forage legume species. Though it has superior quality and yield in the first two years of production, red clover has poor persistence and experiences sharp declines in plant density in later years. This poses a problem, as leys in the region are generally grown for three to four years. Wild, native legume species with longer persistence may be able to provide a legume component to leys in later years of production, while also providing diverse floral resources for pollinators.

The results from this thesis demonstrate that press juicing produced fractions with a better nutrient composition for the intended purpose than leaf stripping when fractionating pure stands of red clover and alsike clover. The higher nutrient value of the juice is mainly due to its lower fiber concentration. More work is still needed to compare these fractionation methods and their resulting fractions. Leaf stripping was also tested on mixed stands of red clover and timothy grass and demonstrated that the leaf fraction produced had a higher nutrient value than the forage if harvested conventionally. Though leaf stripping increased the nutrient composition, it still had large amounts of fiber that would limit its utility as a monogastric feed. Due to the abundance of work already done on press juicing, as well as the superior nutrient composition of the juice, press juicing shows higher potential for integration in the near future.

To identify wild, native legume species, botanical resources were surveyed to select species with promising traits for agricultural production. The use of botanical resources provided a quick way to identify wild species based on key traits of interest. Seven species were identified as having potential for inclusion in leys due to their early flowering times, which would provide important pollinator resources early in the season when flowers are generally lacking. Four of these seven species were grown in mixed leys with timothy grass to evaluate their nutrient composition and potential yield. Two of the species, *Vicia sepium* and *V. cracca*, demonstrated a promising nutrient composition. Additional years of data collection are still required to evaluate the potential of these species during the entire lifespan of a ley.

Populärvetenskaplig sammanfattning

Grovfoder från gräs och baljväxter som odlas för att utfodra boskap är en viktig del av jordbrukssektorn i nordiska länder. Med långvarande dagsljus och milda temperaturer är klimatet i den här regionen idealt för produktionen av grovfoder med optimalt näringsinnehåll. Grovfoder i den här regionen odlas vanligtvis som blandvallar, där gräs och baljväxter samodlas som en del av en växtföljd. Vallar representerar uppemot 75 % av den odlingsbara landytan i norra Sverige och är ett nödvändigt foder i all produktion med idisslare, t.ex. mjölkproduktion. Den här avhandlingen utforskar metoder för att öka diversiteten i vallproduktionen, både i avseende av hur fodret kan användas och vilka baljväxtarter som kan odlas.

Att skapa fler sätt att använda vallfoder är av särskilt intresse i produktionssystem med enkelmagade djur, till exempel grisar och höns. Dessa kräver foder med högt proteininnehåll och minimalt med fiber, eftersom deras förmåga att bryta ned fibrer är betydligt sämre än för idisslare. För närvarande så importeras stora mängder sojabönor för att utfodra enkelmagade djur. Produktionen och importen av sojabönor är inte miljömässigt hållbart och därför kan lokal produktion av proteinrika fodermedel hjälpa till att öka hållbarheten i system med enkelmagade djur. Tidigare studier har utforskat potentialen för fraktionering av vallfoder för att skapa en produkt med mer lämpligt innehåll för enkelmagade och en produkt lämplig för idisslare. Den här avhandlingen fokuserar på två av dessa metoder: 1) pressa juice från vallen och 2) dela upp vallen i blad och strå. Pressningen funkar genom att vallgrödan krossas, pressas och separeras till en flytande del som är rik på protein och en fast rest som är rik på fibrer. Separationen av blad och strå görs maskinellt i fält och skapar två fasta fraktioner, där bladen har högre proteinhalt och strået har högre fiberhalt.

Att öka mångfalden i vallarna med fler arter kan ge lösningar på problemområden som återkommer vid vallodling och kan även öka hållbarheten genom att vallen bidrar med fler ekosystemtjänster. Vallar i norra Sverige inkluderar vanligtvis rödklöver som den huvudsakliga baljväxten. Rödklöver har ofta hög kvalitet och avkastning de första två produktionsåren, men efter det så försämras uthålligheten och planttätheten går skarpt nedåt vid senare år. Detta skapar problem då vallar i regionen generellt odlas under tre till fyra år. Vilda, inhemska baljväxtarter med bättre uthållighet skulle kunna ge en baljväxtkomponent till vallar under senare produktionsår, samtidigt som de kan öka blomresurser för pollinerare i landskapet.

Resultaten från den här avhandlingen visar att pressning av rödklöver och alsikeklöver i renbestånd producerar foderfraktioner med lämpligare näringsinnehåll för ändamålet jämfört med att dela grödan i blad och strå. Det bättre näringsinnehållet är främst i den flytande komponenten från pressningen med den signifikant lägre andelen av fiber. Mer forskning krävs dock för att jämföra dessa fraktioneringsmetoder och värdet av deras slutliga delar. Blandbestånd av rödklöver och timotej testades med metoden som delar grödan i blad och strå. Resultatet visar att bladfraktionen producerade ett bättre näringsvärde än om grödan skördats konventionellt. Trots det begränsar höga fiberhalter fortfarande bladfraktionens användbarhet som foder till enkelmagade. Då det redan idag finns stora mängder forskning publicerat om pressningen och då näringsnnehåll i juicefraktionen är mer lämplig så visar pressningen en högre potential för integration i produktionssystem i den nära framtiden.

För att identifiera lämpliga vilda, inhemska baljväxtarter så utforskades botaniska resurser i syfte att välja arter som påvisar lovande egenskaper för produktion i jordbruk, vilket är ett snabbt sätt att identifiera vilda arter baserat på viktiga egenskaper av intresse. Sju arter identifierades som lämpliga för att inkluderas i vallar genom deras tidiga blomning, vilket skulle ge viktiga resurser till pollinatörer tidigt på säsongen när blommor ofta saknas i jordbrukslandskapet. Fyra av dessa sju arter odlades sedan i vallar blandat med timotej för att utvärdera deras näringssammansättning och potentiella avkastning. Två av dessa arter, *Vicia sepium* och *V. cracca*, visade på en lovande näringssammansättning. Fler år av datainsamling är fortfarande nödvändigt för att utvärdera potentialen i dessa arter under hela vallens livslängd.

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II

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METHODS

Fractionation of mixed grass and clover stands using a leaf stripper

Brooke Micke[1] | Sanna Bergqvist[1] | Steffen Adler[2] | Julien Morel[3] | David Parsons[1]

[1]Department of Crop Production Ecology, Swedish University of Agricultural Sciences, Umeå, Sweden

[2]Department of Grassland and Livestock, Norwegian Institute of Bioeconomy Research, Ås, Norway

[3]Joint Research Center, European Commission, Ispra, Italy

Correspondence
Brooke Micke, Department of Crop Production Ecology, Swedish University of Agricultural Sciences, 901 83 Umeå, Sweden.
Email: brooke.micke@slu.se

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Abstract

Leys are an important part of northern European livestock production, particularly for ruminants since monogastric animals are limited in their ability to digest the fibres of the forage. Crop fractionation methods are a promising option to make forages more beneficial for monogastric animals and decrease the amount of imported protein feed. A leaf stripping harvesting technique was evaluated at Röbäcksdalen in northern Sweden in mixed grass-clover leys over 2 years. The PremAlfa Mini leaf stripper (Trust'ing-Alf'ing, Nantes, France) worked well in mixed stands, harvesting on average a third of the available forage biomass, primarily in the form of leaves and soft stems from the clover plants. It proved successful in producing a forage fraction that had a significantly higher crude protein (CP) concentration (+39.1%) and lower neutral detergent fibre (aNDFom) concentration (−21.4%) than the pre-harvest mixed sward (all significant at $p < .05$ level). Due to the remaining high level of aNDFom in the leaf stripper fraction, it is more suited for use as an energy source for monogastrics rather than as a protein supplement. Alternatively, the leaf stripper fraction could be used to increase digestibility and CP content in the feed rations of high producing dairy cows.

KEYWORDS
biorefinery, crude protein, forage, fractionation, monogastric, neutral detergent fibre, ruminant

1 | INTRODUCTION

As the need for protein feed for livestock has increased, so has the development of new techniques to create alternative, locally produced protein-rich feeds. The EU is heavily dependent on the import of soybean meal as the main source of protein for the livestock sector, but a new emphasis on biorefining of local forages paves the way for the production of a sustainable protein source in Europe (van Krimpen et al., 2013). Forages, particularly forage legumes, are an important protein source for ruminants. Monogastrics, however, require high quality protein with a specific amino acid profile and have limited ability to digest unprocessed forage fibres, thus limiting their usage of forage legume-based feed. Fractionation of forage legumes through biorefinery bypasses these limitations through the creation of a forage-based protein source with a high feed value and a balanced amino acid composition, ideal for monogastrics (Laudadio et al., 2014). Current methods of forage fractionation include sieving, pin milling, air classification, and twin-screw press juicing (Damborg et al., 2018; Laudadio et al., 2014; Wu & Nichols, 2005). Previous studies have shown that the protein-rich fraction created through forage biorefinery can act as a suitable replacement to soybean meal in the diet of chickens (Damborg et al., 2018; Laudadio et al., 2014; Stødkilde et al., 2020; Wu & Nichols, 2005). Additionally, the fibre-rich coproduct can serve as an alternative source of forage for dairy cows, thus increasing the sustainability of the system (Damborg et al., 2018). All fractionation methods mentioned above are post-harvest and require a multi-step process to achieve the end product. An alternative method that allows for fractionation during the harvest process could present a more streamlined approach.

A potential way to achieve harvest level fractionation is to consider how protein and fibre are partitioned throughout the plant. The leaves of forage legumes contain higher levels of soluble protein due to photosynthetic machinery, as well as lower levels of fibre due to their lower cell wall content when compared to stems (Fiorentini & Galoppini, 1983). The concentration of extractable true protein was also found to be higher in the leaf than the stem for both red clover (*Trifolium pratense* L.) and lucerne (*Medicago sativa* L.) (Hakl et al., 2016; Solati et al., 2018). This difference in nutritive value between the leaf and the stem presents an opportunity to fractionate forage legumes through the separation of leaf from stem, creating a protein-rich fraction consisting of leaves and a fibre-rich coproduct from the stems. The high crude protein and low fibre concentrations in leaves makes them a potential protein feed source for monogastrics. Additionally, the stems that remain are high in fibre and could be used as a forage source for ruminants.

The focus of this study is on leaf stripping, a harvest-level fractionation method which separates the leaves, containing easily digestible protein and a low fibre concentration, from the stems (Julier & Huyghe, 1997). Leaf stripping involves the use of harvest machinery that removes a high proportion of the leaves and the soft, upper portion of the stem, while leaving the fibrous portion of the stem behind. The remaining plant material can then be harvested using traditional methods and utilised as a high-fibre coproduct (Figure 1). Previous studies have explored the potential of leaf stripping using harvest machinery either modified or designed specifically for leaf stripping. They have shown promising results for the use of leaf stripping fractionation techniques to improve the nutritive value of the harvested material in comparison to conventional harvesting techniques (Andrzejewska et al., 2020; Liebhardt et al., 2022; Shinners et al., 2007).

Forage production in northern Europe is dominated by leys, in which forages are grown in rotation with annual crops to produce animal feed (Nilsdotter-Linde et al., 2019; Nykänen et al., 2000; Steinshamn et al., 2016). Leys are typically grown as either pure grass or mixtures of grasses, legumes and forbs. In addition to the production of forage for livestock, leys increase agrobiodiversity, sequester carbon, and provide other environmental benefits (Conant et al., 2001; Lemaire et al., 2015). The most prominent forage legume in northern Europe is red clover, which is generally grown in mixtures with various species of grasses, such as timothy (*Phleum pratense* L.), meadow fescue (*Festuca pratensis* Huds.), and perennial ryegrass (*Lolium perenne* L.), among others (Frankow-Lindberg, 2017). Mixed leys of grasses and legumes produce higher yields over time than pure legume stands and a superior nutritive value than pure grass (Finn et al., 2013; Lüscher et al., 2014). As no previous studies have investigated the potential of leaf stripping for mixed stands, it is essential to determine the plausibility of using the machinery and the nutritive value of the resulting fractions. Leaf stripping mixed leys could provide an opportunity to produce local protein feed using typical forage production systems in northern Europe.

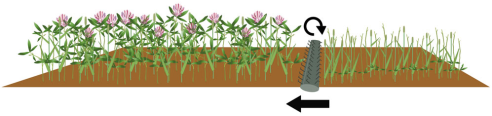

FIGURE 1 An illustration of the leaf stripping method used in this experiment. As the leaf stripper is driven through the plot, it removes leaves and the upper portion of the stem of the legume, which are collected in the machine. The more fibrous legume stem and the majority of the grass remain and are harvested using traditional harvesting methods.

This study is based on the idea that farmers could opportunistically fractionate their leys through leaf stripping in mixed stands with a high percentage of red clover. This may be particularly applicable in the second and third cuts in northern Europe, as the percentage of clover in the stand for these cuts is generally much higher than in the first cut. The following research questions are addressed in this study: (1) Can a leaf stripper machine be used in mixed leys to improve the nutritive value of the resulting fraction when compared to the mixed sward? (2) To what extent does leaf stripping mixed leys of red clover and grass improve the feed value compared to material harvested conventionally? (3) What measurable characteristics of mixed leys affect the nutritive value of the leaf stripper fraction?

2 | MATERIALS AND METHODS

2.1 | Data collection

This study was performed at Röbäcksdalen, a research station located in northern Sweden (63.81° N, 20.24° E). Plots used for sampling were typical mixed ley systems sown with timothy and red clover. Twenty sampling locations were selected each year in 2021 and 2022 ($n = 40$), based on having a visually homogeneous distribution of red clover. Samples were taken throughout the entire season and in swards of different compositions, heights, and phenological stages to obtain a diverse dataset representing a large range of potential nutritive value and yield. To define the sampling area, a four-metre long strip was measured and marked (Figure 2). The normalised difference vegetation index (NDVI) was measured over the length of the entire plot using the GreenSeeker hand-held crop sensor (Trimble, Sunnyvale, California, USA). NDVI is related to the chlorophyll content and leaf area, and thus is a widely used numerical index to evaluate the density and vigour of vegetation. NDVI can be defined as:

$$\mathrm{NDVI} = \frac{\mathrm{NIR} - \mathrm{R}}{(\mathrm{NIR} + \mathrm{R})} \tag{1}$$

where NIR is the reflectance of near-infrared and R is the reflectance of the visible red. Values for NDVI range from −1 to +1, with

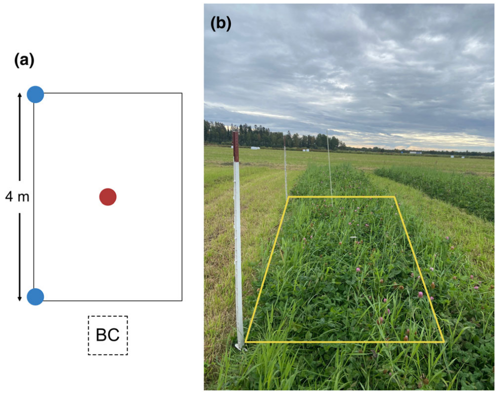

FIGURE 2 Representations of the sampling area: (a) Each blue dot represents a corner of the plot (4 m apart). The red dot represents the hoop used for measurements within the sampling area. The botanical composition (BC) square was placed outside the area used for leaf stripping; (b) The approximate harvested area is represented by the orange polygon. The stick in the middle represents the location of the hoop.

+1 representing an area with the highest possible density of green leaves.

A hoop (76 cm diameter) was placed in the middle of the sampling area and measurements were taken for the height of the tallest clover when stretched and clover phenological development stage (Nadeem et al., 2019). Clover phenological stage was described on a scale from 1.00, which signified first visible leaf, to 4.00, which signified seed formation. Four individual healthy clover plants in the hoop were measured with a Dualex 4 Scientific leaf-clip meter (Dx4, FORCE-A, Orsay, France) to determine the chlorophyll content index (CCI). The Dualex calculates CCI using the function:

$$f(I_o, I) = \left[\left(I_{(850)}/I_{o(850)}\right)/\left(I_{(710)}/I_{o(710)}\right)\right] - 1 \tag{2}$$

where I_o is the signal without the leaf, I represents the signal when the leaf is present in the leaf-clip, and the subscript values correspond to wavelengths (Cerovic et al., 2012). The CCI values presented in this article are the result of transformation using the formula:

$$M = k \times f(I_o, I) + c \tag{3}$$

where k is the proportionality constant used to convert the units to $\mu g\ cm^{-2}$, $f(I_o, I)$ is the result of the CCI function, and c is the constant to correct for model bias (Nauš et al., 2010). Measurements were performed with the adaxial leaf side facing the light source to mitigate for leaf heterogeneity. A location for collecting the botanical composition sample was selected at the end of the sampling strip (Figure 2), measured with the GreenSeeker, delineated with a sampling quadrat (50 cm × 50 cm), and cut by hand at a stubble height of approximately 8 cm. Prior to leaf stripping, the ends of the plot were trimmed with a lawnmower to form clean edges. The hoop was removed from the plot and an additional stick was placed at the edge of the plot, perpendicular to the midpoint of where the hoop had been placed.

The PremAlfa Mini electric leaf stripper prototype (Trust'ing-Alf'ing, Nantes, France) used in the experiment has tines that rotate opposite to the direction of the wheels when in forward motion to separate leaves from stems. The separated leaves are subsequently deposited into a container held within the machine. The harvesting width of the machine is 80 cm. Rotor height, rotor speed, and ground speed were adjusted according to the judgement and experience of the operator, aiming for the machine to operate at the height in the canopy where the majority of leaves was found. The height of the rotor ranged from 9 to 25 cm and on average was set to 14 cm. The rotation speed of the leaf stripper was set to approximately 260–280 rpm, however this dropped to an average of 240 rpm when the machine engaged with the plant canopy. Ground speed was set to approximately 1.7–2.5 $km\ h^{-1}$ and was subsequently recorded as the leaf stripper passed through the plot. The actual ground speed ranged from 0.8 to 2.2 $km\ h^{-1}$, depending on the amount of biomass, slope of the selected plot, and other factors. The ratio of speed of the rotor tines to ground speed was on average 25.6, though was quite variable due to a wide range in ground speed. Harvesting loss was not measured, as visual assessment post leaf stripping indicated that nearly all material harvested by the leaf stripper was successfully collected in the machine.

Following harvest, the residual fraction left by the leaf stripper was sampled using the 50 × 50 cm quadrat placed where the hoop was located for pre-harvest measurements. Residual material within the quadrat was hand harvested at a cutting height of approximately 8 cm. The sampling included 40 independent sampling areas, 20 harvested between the 24th of June and 1st of September 2021 and 20 harvested between the 23rd of June and the 8th of September 2022, representing a typical forage harvest window for northern Sweden.

The material harvested by the leaf stripper was weighed (fresh) and a subsample of 1 kg fresh weight was taken for further analysis. The botanical composition sample was separated into three different fractions: grass, clover, and broad-leaf weeds. Grass weeds were included in the grass fraction. The leaf stripper subsample, botanical composition samples, and residual fraction sample were all weighed for fresh weight, dried at 60°C for at least 48 h until they reached a constant weight, and weighed again, to facilitate calculation of dry matter. The dried samples were milled to pass through a 1 mm screen and stored for chemical analysis.

2.2 | Description of the harvested fractions

The different pre-harvest biomass fractions represented the mixed sward and consisted of the grass fraction (GF), clover fraction

(CF) and weed fraction (WF). The WF was small (the highest value was 5.8%), and thus there was not sufficient material for lab analysis of nutritive value. There were two post-harvest fractions: the fraction harvested by the leaf stripper (LSF) and the residual fraction (RF), which includes the grass, clover stems, and other material not harvested. Thus, the fractions that were assessed for nutritive value included GF, CF, LSF, and RF. Nutritive value of the mixed sward was calculated based on the weighted GF and CF results. Figure 3 shows the fractions in relation to each other.

2.3 | Nutritive value analysis

Dried samples were ground to 1 mm to prepare for chemical analysis. A subsample was re-dried at 103°C for 16 h and cooled in a desiccator before weighing, to determine dry matter (DM) concentration. Amylase-treated, ash-free neutral detergent fibre (aNDFom) was analysed using the method of Chai and Udén (Chai & Udén, 1998). Crude Protein (CP) was analysed using the Kjeldahl-N method, according to the Nordic Committee on Food Analysis (Nordic Committee on Food Analysis, 1976), using the 2520 Digestor, Kjeltec 8400 Analyser unit, and Kjeltec 8460 sampler unit (Foss, Hillerod, Denmark). Organic matter digestibility (OMD) was determined using the rumen degradable organic matter (VOS) method (Lindgren, 1979). Samples were transferred to a glass filter crucible. Rumen fluid from a cow fed a standardised ration at maintenance level with a forage to concentrate ratio of 70:30 was filtered and mixed with a buffer (pH 7, 38°C under anaerobic conditions). Samples were incubated with the rumen fluid-buffer mixture for 96 h. After incubation, the fluid was filtered through a sintered glass disc, washed with deionised water and acetone, and dried overnight. The crucible was then weighed, ashed, and weighed again.

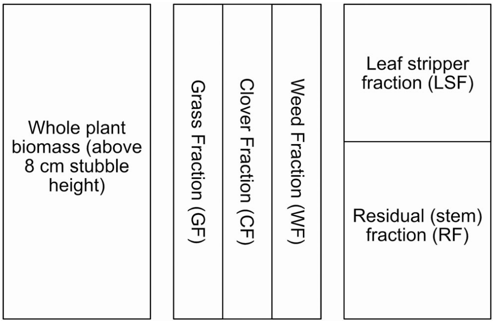

FIGURE 3 Representations of the biomass fractions. The botanical composition sample was separated into the grass fraction (GF), clover fraction (CF), and weed fraction (WF). Following leaf stripping, the relevant fractions were the leaf stripper fraction (LSF) and the residual fraction (RF).

2.4 | Statistical analysis

To assess the differences in nutritive value characteristics, (CP, aNDFom, VOS digestibility, and ash) between different plant fractions, each output variable was analysed using general linear mixed model procedures in PROC GLIMMIX (SAS software version 9.4, SAS Institute Inc., 2008). Plant fraction and year were treated as fixed effects. Denominator degrees of freedom were approximated using the Kenward-Roger method. The RANDOM statement was used for fraction, with sample ID as the subject, using an unstructured covariance structure. Quantile-quantile plots and distributions of studentised residuals were assessed for normal distributions and homoscedasticity. Tukey's statistic was used to test differences ($p < .05$) among means when only the main effect of fraction was significant. When the interaction between fraction and year was significant, the Holm-Bonferroni method was used to test comparisons ($p < .05$) between the same fraction over both years and all fractions within each year.

The Shapiro–Wilk test was used to determine if the data followed a normal distribution (R Studio software version 2022.12.0 + 353, R Core Team, 2022). As the data did not follow a normal distribution, the correlation between each explanatory variable and each response variable was evaluated using the Kendall correlation method (R Studio software version 2022.12.0 + 353, R Core Team, 2022). To build multiple regression models for predicting nutritive value characteristics, PROC GLMSELECT (SAS software version 9.4, SAS Institute Inc., 2008) was used. To estimate the post-harvest nutritive value of the LSF, two multiple regression models were constructed for each variable (CP, aNDFom, VOS digestibility, and CP Yield). One model was constructed using only pre-harvest field measurements (referenced further as field model). The other was constructed using pre-harvest field measurements and pre-harvest nutritive value measurements (referenced further as full model). The explanatory variables are summarised in Table 1. Two datasets were used to construct the eight models outlined above, one with all explanatory variables and another limited to explanatory variables with moderate or strong correlation to the response variable. Variable selection was performed using the STEPWISE option and the PRESS statistic as the criterion, with 0.05 and 0.10 specified as the significance levels for variable entry and removal, respectively. The resultant models were assessed using PROC REG with the PARTIAL option to assess the linearity of partial regression plots. For reporting data, the adjusted r-squared criterion (R^2) was used.

3 | RESULTS

3.1 | Summary of data

The field and nutritive value data collected are summarised in Table 1. The total yield (clover, grass, and weeds) before harvesting is not a focus of this study, as the data are approximations based on a single sample quadrat.

TABLE 1 Summary statistics and description of field-measured variables and of the nutritive value of the pre-harvest sward (mixed, clover, and grass).

Variables	Name	Unit	Mean	Minimum	Maximum	Description
Field-measured	Clover stage		3.3	2.3	4.0	Clover stage of the furthest advanced plant
	Clover fraction	%	62.2	33.3	98.1	Fraction of clover in the sward, on a DM basis
	Grass fraction	%	37.1	1.5	66.6	Fraction of grass in the sward, on a DM basis
	Weed fraction	%	0.75	0.00	5.80	Fraction of weed in the sward, on a DM basis
	CCI	μg cm^{-2}	30.5	25.8	44.0	Chlorophyll content index from Dualex measurement on red clover leaves
	Day of the year		203	174	251	Day of the year starting from January 1st
	NDVI		0.87	0.82	0.91	NDVI measurement taken with the GreenSeeker across the whole length of the plot
	Tallest clover	cm	67.4	48.0	92.0	Clover height measurement from the ground to the top of the longest stretched plant
	Total yield	kg DM/ha	3554	2126	5368	Total yield calculated from the sample taken for analysis of botanical composition
	LSF yield	kg DM/ha	1164	533	2386	Yield of the leaf stripper fraction (LSF), calculated from the area harvested using the leaf stripper.
	RF Yield	kg DM/ha	2390	1376	4532	Yield of the residual fraction (RF), calculated from the area harvested using the leaf stripper.
	LSF in total yield	%	32.2	16.5	57.0	The LSF, as a fraction of total yield, calculated from the sample taken for analysis of botanical composition and the area harvested using the leaf stripper.
Sward nutritive value	CP	g/kg DM	168	90.9	214	Crude protein using the Kjeldahl-N method
	aNDFom	g/kg DM	422	321	517	aNDFom concentration
	Digestibility	g/kg DM	823	757	877	Organic matter digestibility using the VOS method
Clover nutritive value	CP	g/kg DM	186	129	236	Crude protein using the Kjeldahl-N method
	aNDFom	g/kg DM	344	268	422	aNDFom concentration
	Digestibility	g/kg DM	815	670	870	Organic matter digestibility using the VOS method
Grass nutritive value	CP	g/kg DM	136	59.6	231	Crude protein using the Kjeldahl-N method
	aNDFom	g/kg DM	551	485	642	aNDFom concentration
	Digestibility	g/kg DM	830	694	902	Organic matter digestibility using the VOS method

Abbreviations: aNDFom, neutral detergent fibre; CP, crude protein; DM, dry matter; LSF, leaf stripper fraction.

3.2 | Nutritive value of the resultant fractions

The different dry matter fractions, both pre-harvest (clover, grass, and mixed sward), and post-harvest (leaf stripper and residual) had different compositions in terms of nutritive value. All statements of significance are at the $p < .05$ level. For CP concentration, there was a significant interaction between year and fraction (Figure 4a). The CF, GF, and sward were not significantly different to each other in 2021. In 2022, the CF had a significantly higher CP concentration than both the GF and the sward. Following leaf stripping, the LSF had a higher CP concentration than all other fractions in both years. In both years, the RF had a lower CP concentration than the LSF, CF, and sward, but a higher CP concentration than the GF. There was no significant difference between the CP concentrations of the same fraction between years for any of the fractions.

There was also a significant interaction between year and fraction for aNDFom concentration (Figure 4c). All fractions had significantly different aNDFom concentrations in 2021, with the GF having the highest and the LSF having the lowest. In 2022, the aNDFom concentrations of the GF, sward, and RF were significantly higher than the LSF and the CF. The CF and the LSF had the lowest aNDFom concentration in both years. In 2021, the LSF has a significantly lower aNDFom concentration than the CF, whereas in 2022 there was no significant difference between the two fractions. For both years, GF had the highest aNDFom concentration, even higher than the RF. The GF, sward, LSF, and RF had significantly higher aNDFom concentrations in 2022 than in 2021, while there was no significant difference between the CF across years.

As there was no significant interaction between fraction and year for VOS digestibility or ash concentration, results are presented for the pooled 2021 and 2022 datasets. The pre-harvest fractions were not significantly different from each other in regards to the VOS digestibility (Figure 4b). The VOS digestibility of the LSF was significantly higher than all other fractions, while the RF was significantly lower than all fractions apart from the CF. The only fractions with significantly different ash concentrations were the GF and the RF, with the GF being significantly higher than the RF (Figure 4d). All other fractions were not significantly different from the GF or RF.

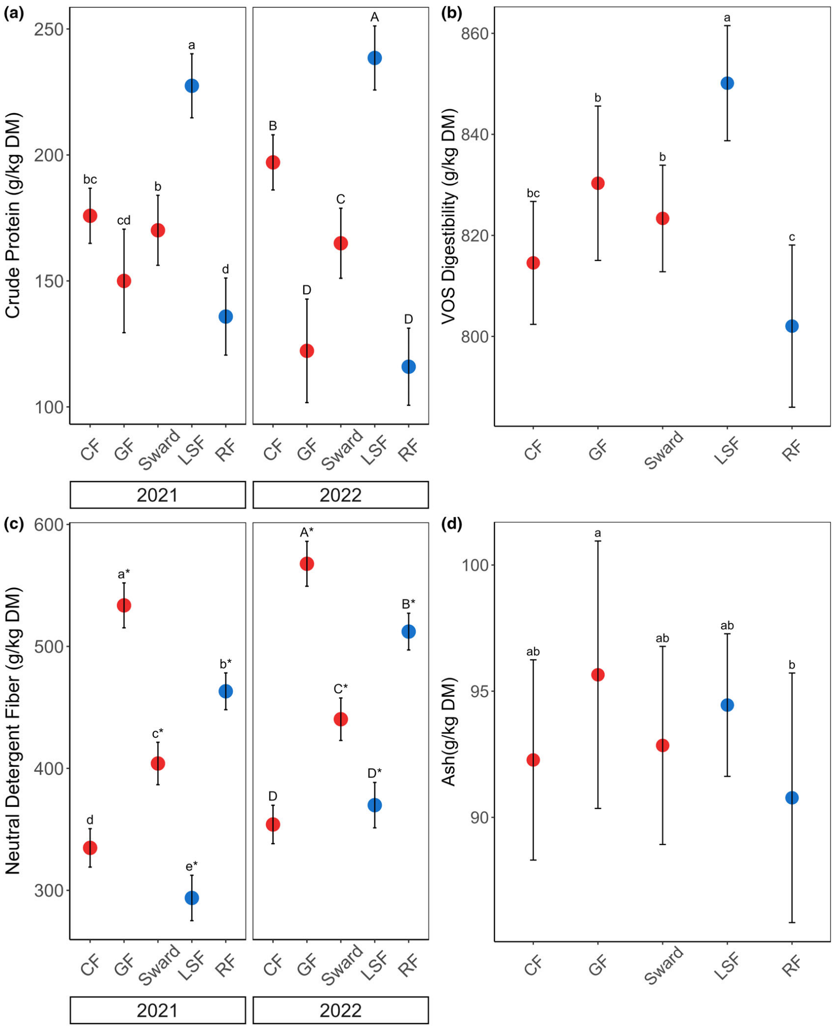

FIGURE 4 Crude protein (a), VOS digestibility (b), neutral detergent fibre (c), and ash (d) of different plant fractions, pre-harvest (red) and post-harvest (blue) using a leaf stripper (LS) in mixed grass-clover leys. Points are least squares means ($n = 20$ for (a) and (c) and $n = 40$ for (b, d). Error bars are 95% confidence intervals. Means with common letters are not significantly different ($p < .05$) according to the Holm-Bonferroni method for subfigures (a) and (c) or Tukey's test for subfigures (b, d). Sub-figures (a, c) present nutritive value data for the interaction of year and fraction. Same fractions across years 2021 and 2022 that are statistically different are denoted with an asterisk. The clover fraction (CF) and grass fraction (GF) constitute the sward pre-harvest. The post-harvest fractions include the leaf stripper fraction (LSF) and residual fraction (RF).

3.3 | Correlations between the leaf stripper fraction nutritive value and the pre-harvest measurements and nutritive value

The level of correlation between explanatory variables (field measurements and nutritive value of the pre-harvest fractions) and the response variables (nutritive value of the LSF) was assessed to identify potential predictors for a regression analysis (Table 2). LSF CP was strongly correlated to the CP of all three pre-harvest fractions. Explanatory variables year, aNDFom of the clover, grass, and sward fractions, and VOS digestibility of the clover and sward fractions were strongly correlated to the aNDFom of the LSF. The LSF VOS digestibility was strongly correlated to the LSF yield, the aNDFom of the CF, and the VOS digestibility of all three pre-harvest fractions. The

TABLE 2 Correlation between nutritive value characteristics (CP, crude protein; aNDFom, neutral detergent fibre; VOS, organic matter digestibility using the VOS method; CP Yield, crude protein yield) of the leaf stripper fraction (LSF) and field measurements and nutritive value characteristics (CP, aNDFom, VOS) of the clover fraction (CF), grass fraction (GF), and sward.

	Response variable			
Explanatory variable	LSF CP (g/kg DM)	LSF aNDFom (g/kg DM)	LSF VOS (g/kg DM)	LSF CP yield (kg DM/ha)
Year	0.147	**0.580*****	*−0.245*	0.129
Clover stage	−0.020	−0.101	−0.171	−0.118
Clover fraction (%)	0.159	−0.144	−0.073	**0.308****
Grass fraction (%)	−0.154	0.154	0.073	**−0.328****
CCI (µg cm^{-2})	0.067	*0.221**	−0.153	0.026
Day of the year	0.093	−0.194	0.081	**−0.383*****
NDVI	0.192	−0.003	−0.035	**0.322****
Tallest clover (cm)	*−0.289***	0.054	*−0.247**	*0.227**
Total yield (kg DM/ha)	−0.122	0.081	−0.110	*0.299***
LSF Yield (kg DM/ha)	−0.077	*0.292***	**−0.301****	**0.841*****
RF Yield (kg DM/ha)	−0.074	*0.228**	−0.176	*0.259**
LSF in total yield (%)	−0.036	0.169	*−0.201*	**0.544*****
CP Clover (g/kg DM)	**0.515*****	0.162	−0.055	−0.003
CP Grass (g/kg DM)	**0.344****	−0.113	0.040	0.036
CP Sward (g/kg DM)	**0.426*****	−0.108	0.065	0.092
aNDFom Clover (g/kg DM)	0.005	**0.369*****	**−0.407*****	**0.374*****
aNDFom Grass (g/kg DM)	−0.115	**0.387*****	*−0.219**	*0.223**
aNDFom Sward (g/kg DM)	−0.192	**0.454*****	−0.194	−0.049
VOS Clover (g/kg DM)	0.023	**−0.336****	**0.527*****	**−0.362****
VOS Grass (g/kg DM)	0.087	*−0.287***	**0.468*****	**−0.385*****
VOS Sward (g/kg DM)	0.072	**−0.344****	**0.561*****	**−0.326****

Note: The correlation coefficients (Kendall's tau) were calculated using the Kendall rank correlation test. Correlation coefficients denoted with asterisks are significant at the levels 0.05*, 0.01**, or 0.001***. Correlation coefficients in bold have strong correlation. Coefficients in italics have moderate correlation. All other coefficients have weak correlation.

response variable LSF CP yield was strongly correlated to many field measurements and pre-harvest nutritive value parameters, totaling 10 of the 21 explanatory variables. All response variables had either strong or moderate correlation to at least one of the response variables.

3.4 | Explanatory variables for nutritive value of the leaf stripper fraction

For all eight models, the dataset using all explanatory variables produced stronger or equal models than the dataset limited to the explanatory variables with strong or moderate correlation to the response variable. All models presented in this article were produced using the dataset with all explanatory variables to maximise fit of the final models (Table 3).

For the full model for CP of the LSF, as expected, the CP of the CF was the most important explanatory variable, explaining 0.56 of the variability. To explain up to 0.67 of the variability, the CP of the sward and the CF percent were also included. In the field model, the variable tallest clover was the first added and explained only 0.15 of the variability. The only other variable included in the model was CF percent, which resulted in an adjusted R^2 of 0.25 for the model.

To estimate aNDFom of the LSF, the field model included only one field measurement, alongside two nutritive value parameters. The aNDFom of the sward fraction was the first variable selected, explaining only 0.47 of the variability. LSF yield and clover CP were added, respectively, to increase the variability explained by the model to 0.69. Similar to the full model, LSF yield was included in the field model and accounted for 0.11 of the variability. The percent CF was then added to increase the adjusted R^2 of the model to 0.31.

The full model estimated the VOS digestibility of the LSF by first including VOS of the sward to explain 0.54 of the variability. Subsequently, total yield was the only other variable included in the model to achieve an R^2 of 0.66. The only field measurement variable included in the full model, total yield, was not included in the field model. The most important explanatory variable for the field model was LSF yield, explaining 0.13 of the variability. The other variable added was clover stage, which only increased the adjusted R^2 of the model to 0.27.

TABLE 3 Multiple regression models ($n = 40$) for predicting nutritive value characteristics (CP, crude protein; aNDFom, neutral detergent fibre; VOS, organic matter digestibility using the VOS method, CP Yield, crude protein yield) of the leaf stripper fraction (LSF). No further variables were added when doing so would not result in a significant ($p < .05$) decrease in the PRESS statistic[†].

Explanatory variable set	Response variable	R^2	RMSE	PRESS	Model
Full	LSF CP (g/kg DM)	0.67	16.2	12,358	50.1 + 0.61(CFCP) + 0.28(SCP) + 36.7(CF)
	LSF aNDFom (g/kg DM)	0.69	30.9	43,360	−272 + 0.96(SNDF) + 0.04(LSFY) + 0.77(CFCP)
	LSF VOS (g/kg DM)	0.66	21.6	21,343	−122 + 1.10(SVOS) + 0.02(TY)
	LSF CP Yield (kg DM/ha)	0.64	72.2	253,543	734–0.87(GFVOS) + 0.06(RFY) + 1.60(CFCP) + 238(CF) − 1.63(DOY)
Field	LSF CP (g/kg DM)	0.25	24.4	26,071	270–1.14(TC) + 63.8(CF)
	LSF aNDFom (g/kg DM)	0.31	46.4	91,959	364 + 0.07(LSFY) − 184(CF)
	LSF VOS (g/kg DM)	0.27	31.5	43,080	989–0.03(LSFY) − 30.4(CS)
	LSF CP Yield (kg DM/ha)	0.49	86.2	337,110	195 + 369(CF) − 1.4(DOY) + 0.05(RFY)

Abbreviations: [†]R^2, coefficient of determination; CF, clover fraction %; SNDF, sward aNDFom (g/kg DM); CFCP, clover fraction crude protein (g/kg DM); CFVOS, clover fraction VOS digestibility (g/kg DM); CS, clover stage; DOY, day of the year; GFVOS, grass fraction VOS digestibility (g/kg DM); LSFY, leaf stripper fraction yield (kg DM/ha); PRESS, predicted residual sum of squares; RFY, residual fraction yield (kg DM/ha); RMSE, root mean square error; SCP, sward crude protein (g/kg DM); SVOS, sward VOS digestibility (g/kg DM); TC, tallest clover (cm); TY, total yield (kg DM/ha).

The datasets used to predict the CP yield of the LSF were modified to exclude LSF yield as an explanatory variable, as it was used to calculate the LSF CP yield. The full model for predicting the LSF CP yield first included GF VOS digestibility to explain 0.36 of the variability. An additional four variables, RF yield, CF CP, CF percent, and day of the year, were also included to explain 0.64 of the variability. The model initially included NDVI of the entire plot, but this variable was dropped by the stepwise process once additional variables were added to increase the adjusted R^2 from 0.63 to 0.64. CF percent was the most important explanatory variable for the field model and explained 0.31 of the variability. After including variables day of the year and RF yield, the model had an adjusted R^2 of 0.49, the highest of all field models.

4 | DISCUSSION

4.1 | Performance of the leaf stripper

The leaf stripper worked well in mixed stands, removing on average a third of the available forage biomass. This is roughly equivalent to 50% of the available clover biomass; however, small amounts of grass were also included in the LSF. The success of the machine in removing clover leaves in mixed stands likely depends on the height and maturity of the plants, as well as machine settings such as the height of the leaf stripper rotor, rotational speed, and ground speed. Proper adjustment of the machine requires the user to observe the composition of the LSF and RF, and make adjustments accordingly. In order to fully understand the performance of the leaf stripper, additional work is needed to determine the proportion of clover leaves collected in the LSF. This could be achieved by hand sorting the LSF and RF post-harvest to calculate the percent of clover leaves collected through leaf stripping. The leaf stripper setting and the biomass of stand, however, will heavily influence these results.

4.2 | Effects on nutritive value

The results clearly showed that the LSF had a significantly higher CP concentration than all other fractions. Following leaf stripping, the CP concentration of the LSF was 39.1% higher than the sward and 25.0% higher than the CF. A previous study comparing conventional harvesting to leaf stripping for pure stands of red clover reported a 32.3% higher CP concentration in the LSF than the clover harvested conventionally, considerably higher than the results presented here from the mixed stands (Liebhardt et al., 2022). The smaller increase in CP concentration of the LSF compared to the CF seen in our results can be attributed to the inclusion of grass in the LSF fraction. As the CP concentration of the GF was 27.0% lower than the CF, the inclusion of grass in the LSF decreases its CP concentration. Although higher CP concentrations could be achieved when leaf stripping pure clover stands, mixtures of grass and clover are preferable in northern Europe due to their higher yields, longer persistence, and increased sustainability.

The impact of leaf stripping on aNDFom was less consistent however. The LSF had a significantly lower aNDFom concentration than both the sward (−110 g/kg DM) and the CF (−41 g/kg DM) in 2021. In 2022, however, the aNDFom of LSF was only significantly lower than the sward (−70.5 g/kg DM), as the aNDFom of the LSF and CF were not significantly different. The aNDFom concentration of the LSF was significantly higher in 2022 than in 2021 (Figure 4). Considering the LSF is made up of clover leaves and grass and that the aNDFom concentration of the CF was not significantly different between 2021 and 2022, the change in aNDFom concentration of the LSF must be explained by the changes in the GF. For the GF, the aNDFom concentration (+34.1 g/kg DM) and the sward percentage (+7.48%) increased in 2022 compared to 2021 (Figure 4). This higher aNDFom concentration and increased amount of grass in the sward likely contributed to the higher aNDFom of the LSF in 2022. Though not included in the analysis due to missing data, the grass stage at harvest could also have contributed the difference in aNDFom concentration between years. Previous studies on leaf stripping have only been done in legume monocultures and thus the results are not directly comparable when considering pre-harvest fractions and their influence on the LSF. An experiment performed in pure lucerne reported that the RF contained the highest concentration of aNDFom compared to the LSF and whole lucerne plant (Sikora et al., 2019). This trend is also seen in the data presented here, as the RF contained a higher aNDFom concentration than both the LSF and CF (Figure 4).

Leaf stripping had a significant effect on the VOS digestibility when compared to the sward and the CF (Figure 4). The LSF had 3.25% higher VOS digestibility than the sward and 4.37% higher than the CF. Leaf stripping had a small effect on ash concentration, with the only significant difference being between the GF and the RF (Figure 4). Based on the improved nutritive value of the LSF compared to the CF and sward, the largest effect of the leaf stripper was increasing the CP concentration of the resultant product, while decreasing the aNDFom concentration and slightly increasing the VOS digestibility were secondary effects.

4.3 | Potential use of the forage fractions

The higher CP and lower aNDFom concentrations in the LSF fractions compared to the sward increases the feed value for monogastrics such as pigs. Pigs can utilise some amount of forage, which can be beneficial for gut health. The CP concentration in the LSF in this study averaged 23% DM, significantly lower than the CP contained in soybean meal which ranges from 45% to 50% DM (Sauvant et al., 2004). The successful integration of the leaf stripper machine into current production systems will require reliable methods of preservation. Though the nutritive value of the LSF is significantly better than that of the mixed sward, the ensiling process has been shown to result in protein degradation, leading to silage with protein concentrations too low to serve as a protein feed (Renaudeau et al., 2022). Due to this, it is not reasonable for the preservation process to be overly expensive. The average moisture content in the LSF (202 g/kg DM) was similar to that of the GF (217 g/kg DM). On-field wilting of leaf material is not possible, thus requiring the inclusion of grain or additives during the ensiling process to achieve an adequate DM content. Previous studies have shown potential for inclusion of crushed barley grain or ground corn to achieve a suitable moisture content for ensiling (Renaudeau et al., 2022; Shinners et al., 2007). Alternatively, formic acid has been successful in inhibiting clostridial fermentation when ensiling forage leaves with a high moisture content (Muck et al., 2010; Shinners et al., 2007). Due to the post-ensiling nutritive value of leaf stripped material, Renaudeau et al. (2022) concluded that legume leaf silages should be considered as an energy source rather than a protein source for pig feeding. If the goal is to develop a protein feed for pigs, further processing (such as juicing of the LSF) to increase protein content and remove fibre may be necessary.

The LSF has similar characteristics to typical forages harvested for ruminants, but with higher protein and digestibility, and lower fibre concentrations. It could be used to increase digestibility and CP content in the feed rations of lactating dairy cows, potentially reducing the need for grain-based concentrates. However, increased CP from clover in dairy diets will not necessarily increase production. The digestion of red clover protein in dairy cows is limited in the utilisation of the protein flowing from the rumen to the small intestine (Vanhatalo et al., 2009), and thus cows fed silages high in red clover may not fully utilise the protein for productive purposes, at least partially due to the presence of polyphenol oxidase (Lee, 2014). The RF, with lower CP and digestibility could potentially be fed to dry dairy cows, heifers, or other ruminants requiring feed with less energy and CP.

4.4 | Modelling nutritive value of the leaf stripper fraction

The purpose of the modelling component was to assess whether there are measurable characteristics of mixed leys that affect the nutritive value of the LSF. In general, the models for CP, aNDFom, VOS digestibility, and CP Yield of the LSF were quite poor and, based on these results, are not useful methods for assessing the potential nutritive value of the LSF pre-harvest. Although the full models were able to explain on average 70% of the variability, they relied heavily on the nutritive value of the pre-harvest fractions, data that is not typically available prior to harvest. The results of the field models give a better picture of the prediction potential one might have pre-harvest. For LSF CP, aNDFom, and VOS digestibility, the field models only explained on average 28% of the variability. The model for LSF CP yield was able to explain 49% of the variability, though this parameter is directly correlated to the amount of biomass in the field and thus easier to estimate pre-harvest.

Surprisingly, the variables NDVI and CCI were not included in any of the models. Considering these variables represent vegetation greenness and chlorophyll concentration (Cerovic et al., 2012; Tang et al., 2022), one might have expected them to be better indicators of CP concentration. The NDVI reading from the GreenSeeker contains information about the leaf area of the canopy and the chlorophyll content of the measured area. These variables can be highly correlated, especially in the case of non-stressed conditions. Moreover, NDVI is known to be prone to saturation for high levels of biomass (Mutanga & Skidmore, 2004), that is, the vegetation index cannot account for changes in biomass or chlorophyll content. Saturation results in a limited NDVI range (Sharma et al., 2015), which is consistent with the small amount of variability in the NDVI readings between plots in this study, regardless of their differences in yield and botanical composition. The CCI data obtained with the Dualex did not accurately represent the chlorophyll content of the LSF, as the Dualex leaf clip was only used on clover leaflets. The LSF is made up of clover leaflets, petioles, and stems, as well as grass, and thus the CCI data would need to take into account the chlorophyll content of all components of the LSF to provide an accurate indication of its CP concentration. Improving field models could potentially be achieved by the inclusion of additional equipment, capable of predicting nutritive value. Field spectrometers have been shown to have success in estimating nutritive value (Morel et al., 2022; Zhou et al., 2019), however currently the price is an obstacle for practical application. Alternatively, NIR sensors or spectrometers mounted to the harvest machinery, such as John Deere's HarvestLab or Zeiss' Corona extreme, could allow for continual adjustment of leaf stripper settings based on nutritive value measurements in real time.

The botanical composition of the sward, represented by the CF percent in this analysis, can be an important factor in determining the nutritive value of the LSF fraction and was included in three of the four field models (Table 3). Though not done in this study, a botanical separation of the LSF could provide additional information about how much grass the leaf stripper harvests. Previous leaf stripping studies have focused on hand or air separation of the LSF to gain insight into leaf proportion of the LSF. The only published study on leaf stripping of red clover showed that in pure red clover stands, 82% of the LSF was comprised of red clover leaves (Liebhardt et al., 2022). Understanding this mechanism will be essential in understanding the makeup of the LSF, as well as its nutritive value.

4.5 | Further development

This study was an initial investigation of using a leaf stripping machine designed primarily for lucerne in mixed stands of red clover and grass. It is clear that the PremAlfa Mini was suitable for fractionation of red clover in mixed stands. This was evident from visually assessing the resultant LSF, and from the clearly significant differences between the nutritive value parameters of the fractions.

Nevertheless, further investigation is needed to build up a database of samples and accompanying agronomic data. Increased understanding of how the machine functions with changing levels of clover content and increasing levels of biomass is necessary to develop machine setting recommendations based on stand characteristics to ensure consistent efficiency in fractionation. The machine performance likely impacts the resulting nutritive value and yield of the LSF, thus maintaining consistent machine settings across diverse stands will be essential in ensuring a homogenous end product. Variables such as the ratio of rotor speed to forward speed and location on the plant in which the tines fractionate should be further investigated to determine appropriate settings for the intended LSF composition. Additionally, further machine modification may be necessary to optimise fractionation in mixed stands. With further development, it could be possible to suggest the optimal rotor height based on the height of the sward and the botanical composition. The rotor speed when using a full-scale leaf stripping harvester would likely be less influenced by increased biomass due to increased available power, so these issues may not persist once shifting to large scale leaf stripping.

Further processing of the LSF could help achieve a more suitable CP and aNDFom concentration for utilization as a monogastric protein feed. Fractionation of forages through twin screw-press juicing has shown great promise in northern Europe to produce protein feed with suitable nutrient composition for monogastrics. The combination of these two fractionation methods could potentially produce a concentrated protein-feed product high in protein and low in fibre for monogastrics. Based on results of previous studies, juicing of the LSF could achieve a product with a significantly lower fibre content than leaf stripping alone (Colas et al., 2013; Digman et al., 2013; Hansen et al., 2022; Jørgensen et al., 2022).

5 | CONCLUSIONS

This study showed that the PremAlfa Mini leaf stripper machine could successfully separate clover leaf from clover stem and grass in mixed stands. The leaf stripping process increased CP concentration and digestibility, and reduced aNDFom concentration, in comparison to the original sward. The resultant nutritive value of the LSF signifies that it is more suitable as an energy source rather than a protein source for pig feeding. The LSF could however be used to upgrade the nutritional content of forages used for selected ruminants and offer feeds of different nutritive value to classes of animals with different nutritional requirements. The regression models developed to identify measureable characteristics that impact the nutritive value of the LSF are likely not useful for prediction at their current stage. Further development is needed to determine if additional spectrometer measurements can improve the ability of models based on pre-harvest data to predict the nutritive value of the LSF.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Brooke Micke https://orcid.org/0000-0003-4680-5288

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RESEARCH ARTICLE

Using botanical resources to select wild forage legumes for domestication in temperate grassland agricultural systems

Brooke Micke[1] · David Parsons[1]

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Abstract

The need for better understanding and conservation of wild plant resources with potential for domestication or utilization in crop improvement has been highlighted in recent years. Botanical resources such as herbaria, databases, and floras offer an information-rich platform from which to select species of interest based on desirable traits. To demonstrate the potential of these resources, wild, native forage legumes were screened for inclusion in northern Swedish grassland agricultural systems (leys). The poor persistence of red clover in multi-year leys is a limitation to the current management strategy in the region. Wild, native forage legumes with the potential for longer persistence were considered for inclusion as minor components in leys to contribute to the system in later years of production. Using the Umeå University Herbarium, local floras, and both regional and international biodiversity databases, seven wild forage legume species were selected based on phenology, morphology, and native range. Particular focus was given to the potential for species to provide pollinator resources early in the season, leading to species with early flowering being preferred. Biodiversity databases were also used to locate wild populations of the selected species to facilitate seed collection for future cultivation, as additional study of the agronomic potential of the selected species is necessary. Here, we have shown that the rich biodiversity data stored in botanical institutions can jumpstart the selection of wild species for utilization in the agriculture sector based on various traits of interest.

Keywords Forage legumes · Crop wild relatives · Herbaria · Ecosystem services · Fabaceae · Crop candidates · Sustainable agriculture

1 Introduction

Globally, it is estimated that there are nearly 400,000 species of flowering plants (Pimm and Joppa 2015). Throughout the last 12,000 years, humans have domesticated about 2500 of these species, though only 250 are considered to be fully domesticated (Dirzo and Raven 2003; Gruber 2017; Fernie and Yan 2019). Considering that upwards of 50,000 plant species are considered edible, a large gap exists between currently domesticated crops and their wild progenitors that may have potential for cultivation (Warren 2015). The potential of wild plant species is particularly relevant when current crops fail to fit the agricultural systems in which they are integrated. Wild species with agronomic potential may be able to improve the suitability of crop systems to a changing environment and alternative agricultural practices through their unique adaptations to their native region.

Botanical resources are a greatly underused avenue of research into wild species that have agricultural potential. Globally there are over 3000 botanical gardens with herbaria that house an estimated 390 million plant specimens (Miller et al. 2015; Thiers (updated continuously)) (Fig. 1).

Additionally, numerous botanical databases compile data on the taxonomy, morphology, phenology, and ethnobotanical use of nearly all described plant species (Missouri Botanical Garden.; Kattge et al. 2020; Molina-Venegas et al. 2021). These data compiled over centuries provide the perfect platform for the study of crop wild relatives. Two of the world's most prominent botanical gardens, the Missouri Botanical Garden and the Royal Botanic Garden Kew, have initiated projects focusing on the identification and

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✉ Brooke Micke
brooke.micke@slu.se

David Parsons
david.parsons@slu.se

[1] Department of Agricultural Research for Northern Sweden, Swedish University of Agricultural Sciences, 901 83 Umeå, Sweden

FLORA SUECICA
Lathyrus japonicus Willd. ssp. maritimus (L.) Ball
Provins: Ångermanland
Socken: Hörnefors
Lokal: Snöan.
Rikligt på sand/grus innanför Järnhällan.
Altitud: 3 m
UTM 50 km: DR 2
Rikets 5 km: 19 X 90
Datum: 1983-VIII-17
Leg: STEFAN ERICSSON nr:2239
Det:

Fig. 1 Herbarium specimen from the Umeå University Herbarium of *Lathyrus japonicus* L. subsp. *maritimus* (L.) P.W. Ball collected in Ångermanland, Sweden in 1983. Data on plant morphology, phenology, and range can be extracted from specimens such as this. Photograph by Brooke Micke.

conservation of crop wild relatives within the last 10 years (Dempewolf et al. 2014; Ciotir et al. 2019). These projects serve as models for the utilization of botanical knowledge in the field of crop wild relative research.

The legume family (Fabaceae) contains over 19,500 species, making it the third largest family of flowering plants (Azani et al. 2017). Though much biodiversity exists in the family, only 65 species are commercially important and traded globally, 50 of which are forage legumes (Howieson et al. 2008; Kulkarni et al. 2018; Schlautman et al. 2018). This discrepancy suggests that some species with unique adaptations to their native environment and great potential for cultivation may have been overlooked for use in agriculture. Perennial forage legumes are an essential source of protein in sustainable livestock production throughout Europe and the rest of the world. Through their ability to fix atmospheric nitrogen and subsequently contribute usable organic nitrogen into the crop system, legumes increase the sustainability of feed and food production (Carlsson and Huss-Danell 2003). In Europe, the main perennial legume forages planted for harvesting are red clover (*Trifolium pratense* L.) and lucerne (*Medicago sativa* L.), while white clover (*Trifolium repens* L.) is the most commonly sown for grazing (Halling et al. 2004; Geleta et al. 2019). Considering the diversity of wild legumes in the region, increasing the agrobiodiversity of forage legumes has the potential to improve the adaptability and sustainability of forage production.

The potential for new forage legume species is of particular interest in forage production in northern Sweden. Leys, a system in which forages are grown for animal feed as a break from annual crops in a rotation, play an important role in food production systems in northern Sweden, as they provide the forage necessary for dairy and meat production (Kipling et al. 2016). In Västerbotten and Norrbotten, the two northernmost provinces in Sweden, leys made up 68% and 75% of the total arable land in 2021, respectively (Jordbruksverket 2021). Leys are generally harvested two to three times per season for three to four years in the north, though are sometimes harvested for up to eight years before being resown to an annual grain crop (Ericson 2018). In northern Sweden, these leys are generally multispecies swards, containing various grass species and red clover as the dominant forage legume species. Though its productivity is unmatched in the region during the first two years, issues with root rot and clover rot negatively impact its persistence and therefore its yield in the long term (Frankow-Lindberg et al. 2009; Marshall et al. 2017). Solving issues with root and clover rot are challenging and have not been fully resolved through the breeding of disease resistant clover varieties or the application of chemical agents. In addition to the reduction in forage yield following the loss of red clover, the ley productivity is also hindered due to the decrease in biological nitrogen fixation (Riesinger and Herzon 2010). Ley persistence must be increased to fit the current management strategy in the region. A potential solution to this may be the inclusion of wild forage legume species with longer persistence that would continue to contribute fixed nitrogen to the ley system for the later years of production. These wild legumes, when grown as minor components of a ley alongside red clover and forage grasses, could help solve ley persistence issues after year three while still maintaining yields in years one and two. A study on 26 species and four subspecies of native legumes in Sweden demonstrated nodulation in all 30 taxa (Ampomah et al. 2012). These results likely signify that the evaluated species are capable of fixing nitrogen in their native environments. Further study on nitrogen fixation and nodulation of new forage legume species will be essential in evaluating their persistence.

The inclusion of alternative native legumes and the consequent increase in biodiversity in leys can act not only to improve their persistence, but also to contribute additional ecosystem services to create more sustainable agricultural systems (Bianchi et al. 2013). As monocultures have the largest negative impact on pollinators, an increase in agrobiodiversity can act to alleviate some of this threat by providing diverse food sources (Wratten et al. 2012). When managed with biodiversity in mind, grassland systems have the potential to house high levels of plant diversity and thus pollinator resources. Additionally, higher plant diversity in leys would help ensure pollen and nectar sources for pollinators throughout much of the season, with early flowering species being vital due to the lack of floral resources early in the season, particularly in northern Sweden (Johansen et al. 2019).

In an effort to demonstrate the potential for utilizing botanical resources in agricultural research, we used Sweden's major botanical databases and the Umeå University Herbarium to select candidate species of native forage legumes from northern Sweden based on characteristics such as morphology, phenology, range, and habit.

2 Materials and methods

2.1 Study system

Fabaceae is distributed throughout Sweden, with a total of 25 genera and 84 species that are native or naturalized in the country (Krok et al. 1994). All 84 species are placed within the subfamily Papilionoideae DC., the largest legume subfamily with 503 genera and ca. 14,000 species (Azani et al. 2017). In Papilionoideae, leaves are pari- or imparipinnate to palmately compound, but also commonly uni- or trifoliate and leaflets can be modified into tendrils. Following the name, the corolla is typically papilionate, with an adaxial standard petal, two lateral wing petals, and two abaxial keel petals. Root nodules, either indeterminate or determinate, are prevalent in the subfamily, with nodulation occurring in roughly 90% of the genera (Tutin et al. 1968; Sprent 2001; Azani et al. 2017).

2.2 Initial selection criteria

As the focus of this study was to select candidate species that could be grown in northern Sweden, a list of species found in the northernmost provinces was extracted from the Swedish Virtual Herbarium (http://herbarium.emg.umu.se/). The faunistic provinces included were Lycksele Lappmark, Norrbotten, Pite Lappmark, Västerbotten, Ångermanland, and Åsele Lappmark (Johansson and Klopfstein 2020). Based on herbarium records, 79 species representing 25 genera had been collected in these six northernmost provinces. As the specimens were collected over a range of roughly 215 years, an initial taxonomic check using the Tropicos database (https://www.tropicos.org/home) was done to ensure species names were still valid. Four of the specimen names were now invalid at the species level, as they had been reclassified as subspecies or were now considered synonyms to valid species. The remaining 75 species were then used as the initial candidate species list.

Important characteristics were considered as selection criteria to narrow down the list of candidate species. As the goal of this study was to select native legumes that could be grown in leys, species must be native to northern Sweden, be perennial to survive throughout the lifespan of the ley, and have an herbaceous (non-woody) habit to enable conventional harvest and comparable quality to existing species. Self-regenerating annual species were not considered, as the short growing season in northern Sweden combined with multiple harvest management strategies limit the ability for species to set seed. Information on native range, growth duration (i.e., annual, biennial, or perennial), and habit for the candidate species was extracted from the International Legume Database and Information Service (ILDIS) (https://ildis.org/LegumeWeb/). Local flora were also consulted for the collection of these data (Mossberg et al. 1992; Krok et al. 1994). Though not a selection criterion, ethnobotanical data were also collected from ILDIS for each species to gain insight on previous uses. Additionally, occurrence was considered important, as populations would need to be easily located and abundant enough to support seed collection. To ensure this, a list of all specimens from the Swedish Virtual Herbarium of the 75 initial candidate species was compiled, and only species with a minimum of 20 herbarium specimens collected were considered. The list of candidate species was then narrowed to include only those that met the above-mentioned criteria.

2.3 Herbarium selection and measurements

Species from the narrowed candidate list were then evaluated for additional characteristics using herbarium specimens from the Umeå University Herbarium. All specimens available for each species from the Västerbottens län collection were used for measurement and data acquisition. For each specimen, the location of collection, accession number, collector, collector number, and latitude and longitude of collection site were recorded. Traits measured included leaf length, leaf width, and plant height (only for specimens with roots and terminal bud). Leaf length was measured for the compound leaf from the leaf base to the leaf apex of the final leaflet(s). Leaf width was measured at the widest point of the compound leaf. The inflorescence location was noted, as well as whether or not the plant was in flower or fruit on the date of collection. A range of flowering period was constructed using the collection date of all specimens in flower. Final candidate species were selected based on flowering period, as the project was particularly interested in early flowering species.

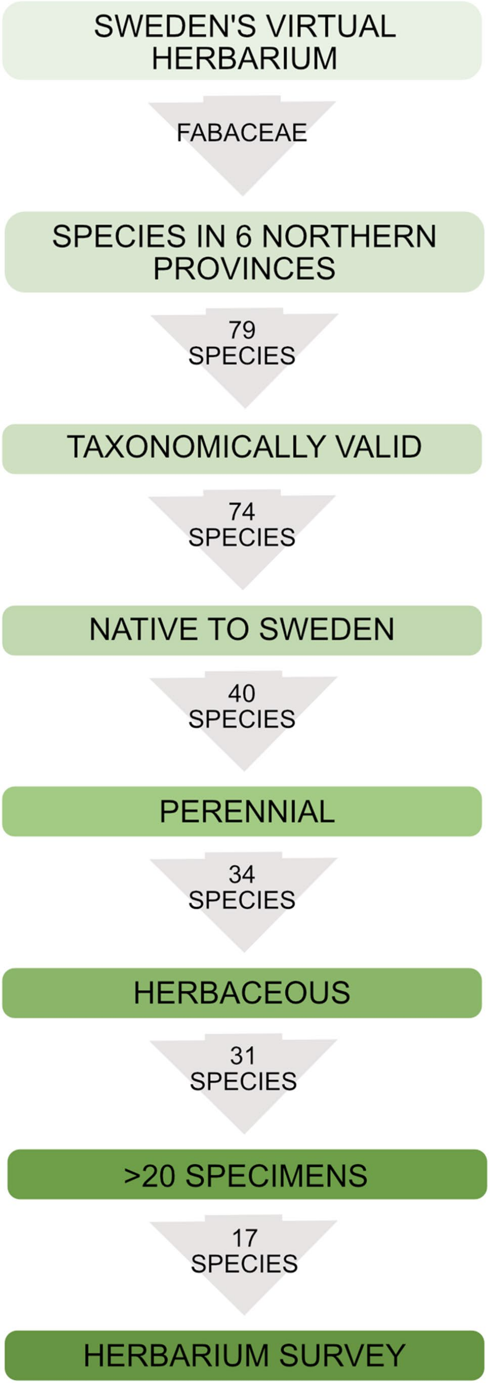

Fig. 2 Flow chart of the initial selection process starting with the ▶ extraction of all Fabaceae species collected in Sweden from the Swedish Virtual Herbarium and ending with the selection of 17 species to be further evaluated in the herbarium selection stage.

2.4 Data analysis

Descriptive statistics of flowering day of year were calculated for each species. Using the minimum and median value for each species, descriptive statistics were then calculated for the combined dataset of all 17 species. Species which were within the minimum and the first quartile of minimum or median flowering day for all species were selected as the final candidate species.

2.5 Selection of wild populations and seed collection

Once the list of candidate species was finalized, the collection of seed from wild populations of each species was planned. As the major objective of this project was to study the quality and establishment of these species when grown in a ley, it was essential to acquire seed for each species. As seed from the selected species is not commercially available and the seed quantity needed for future experiments was not available from gene banks, it had to be collected from populations in the region. Populations of the final candidate species were identified using Artportalen, a database run by the SLU Species Databank within the Swedish University of Agricultural Sciences that is used to report species observations in Sweden (https://artportalen.se/). Several populations of each species were selected and visited in June to July 2020. During this initial visit, sites for future seed collection were selected based on population size. Large populations were selected, as they could accommodate seed collection without endangering the health of the natural population through over collection.

Selected populations were monitored throughout the summer to track seed pod maturity. Once mature, seed pods were collected, allowed to fully dry and then threshed. Seeds were then counted and weighed to determine a thousand seed weight and stored in cool, dry conditions.

3 Results and discussion

3.1 Species selection

Information on the habit, growth duration, and native range of the initial candidate list of 75 species was gathered. Of the 75 species, 66 were herbaceous, 54 were perennial, and 40 were native to Sweden. A count of collected herbarium specimens for each species showed that 36 of the candidate species had more than 20 specimens documented in the Swedish Virtual Herbarium (Fig. 2, Supplementary Material

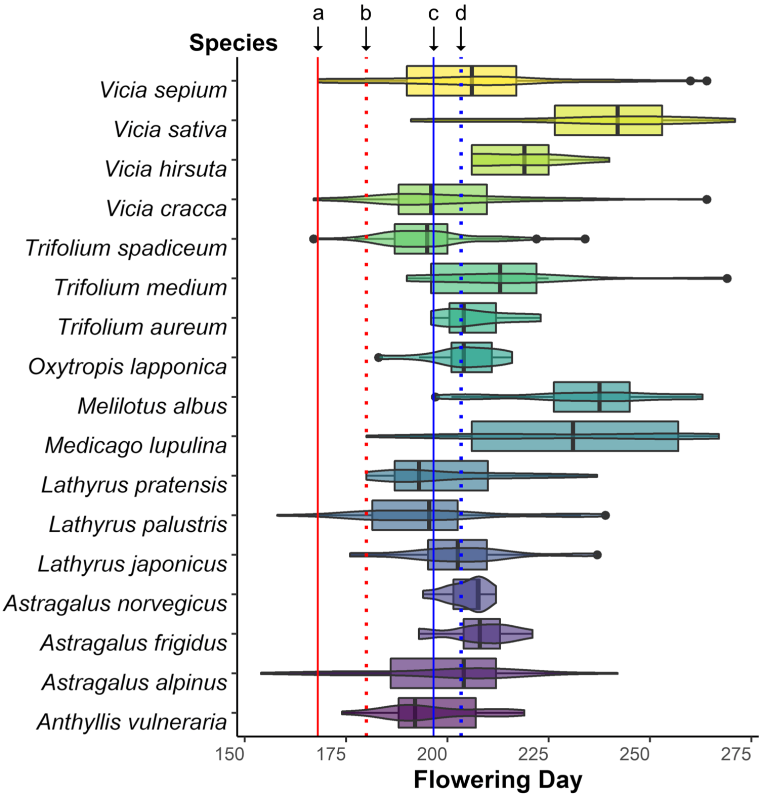

Fig. 4 Flowering day of the herbarium specimens measured. The day of flowering is expressed as *x* of 365, to represent the day of the year out of the total 365 days. Line a represents the first quartile of the earliest flowering day, b represents the median of the earliest flowering day, c represents the first quartile of the median flowering day, and d represents the median of the median flowering day. Left edge of rectangular plot represents the 1st quartile, black vertical lines represent the median, and right edge represents the 3rd quartile. Black points represent outliers.

agricultural landscapes that are often lacking in floral diversity (Decourtye et al. 2010). A study on both stable and declining bee species in Europe showed that red clover pollen was the most commonly collected for half of the studied species (Kleijn and Raemakers 2008). This was likely due to the abundance of red clover grown as a forage crop in the studied regions. With forages having such a large impact on pollinator diet, the diversification of forage legume species in cropping systems has the potential to provide pollinator resources throughout the entire season.

The management of grassland systems, such as leys, has a major impact on pollen resources. The time and frequency of harvest impact floral diversity, with variable harvest times providing the most continuous supply of floral resources (Johansen et al. 2019). Selection of wild forage legume species should be influenced by the management strategy of the system in which they are to be added. The species selected in this study exhibit relatively early flowering times, as they are intended for inclusion in leys, which can lack floral resources early in the season due to the harvest of red clover prior to its flowering. This potential increase in floral diversity early in the season may in turn increase pollinator diversity, as species with foraging activity early in the season will have greater access to resources (Decourtye et al. 2010; Johansen et al. 2019). The impact of these floral resources will greatly depend on the management regime of the ley. Various harvest frequencies and times should be assessed with these potential wild forage legumes to determine whether they are best included in low- or high-intensity systems.

Selecting persistent species was the other main goal of the project, but data was not available on the persistence of the species when grown in an agricultural grassland system. Due to this lack of data, only flowering date was used to narrow down the candidate list during the herbarium survey portion of the selection process.

3.2 Issues with selection

Leaf width and height were measured for each specimen to gain insight on leaf area, as leafiness is an important factor to consider when considering nutritional quality of a plant (Table 1). This parameter was not included in the selection of the final candidate species, as more focus was given to early flowering than leaf area.

Leaf area can be an important trait when selecting wild forage species for use in production, as the leaf:stem ratio of forages can be indicative of their quality. Plants with higher leaf:stem ratios at harvest are generally higher in crude protein and digestibility and lower in NDF (Terry and Tilley 1964; Kalu et al. 1988, 1990). Even with a high leaf:stem ratio, it is unlikely that any of the selected wild species would have a forage quality or yield that could compete with red clover. Current red clover cultivars have been bred extensively to maximize yield and quality, thus making it nearly impossible to replace them with wild species (Geleta et al. 2019). An alternative is to include these wild species only as minor components in leys. In doing so, the yield and forage quality of the wild species must only be high enough not to significantly decrease the yield and quality of the forage harvested. In place of contributing through yield and quality, these wild species may contribute to the system through increased ecosystem services and persistence.

Initial plans also included plant height as an important characteristic, as small plants would be easily outcompeted for light by other species in the ley mixture. This selection criterion was removed, as there is a potential bias towards collecting smaller specimens of a species that will easily fit on a herbarium sheet. Plant height ranges collected from the specimens did not match the ranges found in the Flora Europaea, with the mean plant height from herbarium specimens often being at the minimum end of the range given in the flora (Tutin et al. 1968). Biases in herbarium collections have been documented in several categories, including spatial, temporal, trait, phylogenetic, and collector bias (Moerman and Estabrook 2006; Blonder et al. 2012; Daru et al. 2018). Most of these come about inadvertently to ease collection difficulties or focus efforts on plants of most interest to the collector. The potential of these biases must be acknowledged when utilizing herbarium specimens to draw conclusions about species morphology, phenology, and occurrence. Though these potential biases may skew specimen data when compared to wild populations of a species, herbaria still offer the most extensive collection of plant diversity data available and should be considered invaluable resources in agrobiodiversity research.

The accuracy of data taken from botanical databases must also be considered when extracting data for selection. *Melilotus albus*, *Trifolium aureum*, *Trifolium spadiceum*, and *Vicia sativa* were listed as perennial in ILDIS but mentioned in other databases and floras as annual or biennial (Ciotir et al. 2019; Tutin et al. 1968; Roskov et al. 2005; POWO 2022). After discussing the species with local experts, it was determined that these species do not have perennial habits in northern Sweden. Data were still collected for these species, and *Trifolium spadiceum* was initially selected as a final candidate species. Following the first visit to plant populations of *T. spadiceum*, the inclusion of the species was in question due to plant height. The plants at each population measured under 20 cm and thus would have difficulty competing with other species in the ley mixture. Considering both the lack of perenniality and the plant height, *T. spadiceum* was removed from the final list of candidate species. *Lathyrus japonicus*

Table 1 Median values of data collected from herbarium specimens of the 17 candidate species. The NA present in the median fruit date signifies that no specimens measured in the survey were in fruit.

Species	Median leaf length (cm)	Median leaf width (cm)	Median plant height (cm)	Median inflorescence count	Median fruit date (*x* of 365)
Anthyllis vulneraria L. subsp. *lapponica* (Hyl.) Jalas	4.05	2.55	22.4	13	230
Astragalus alpinus L. subsp. *alpinus*	8.90	2.20	21.1	3	209
Astragalus frigidus (L.) A. Gray	6.05	3.95	17.5	2	220
Astragalus norvegicus Weber	6.10	2.45	25.9	3	208
Lathyrus japonicus L. subsp. *maritimus* (L.) P.W. Ball	9.40	7.20	36.0	2	207
Lathyrus palustris L.	5.05	7.95	39.9	3	223
Lathyrus pratensis L.	2.50	5.20	35.5	2	220
Medicago lupulina L.	2.60	2.20	25.0	13	238
Melilotus albus Medik.	3.65	3.05	34.9	16	240
Oxytropis lapponica (Wahlenb.) Gay	5.35	1.60	11.7	3	221
Trifolium aureum Pollich	2.40	2.80	28.1	6	NA
Trifolium medium L.	10.6	6.80	35.0	2	269
Trifolium spadiceum L.	3.20	2.40	21.6	5	NA
Vicia cracca L.	5.50	3.45	30.7	3	203
Vicia hirsuta (L.) Gray	5.30	3.00	27.1	12	251
Vicia sativa L. subsp. *sativa*	8.70	3.90	45.6	2	222
Vicia sepium L. subsp. *montana* (W.D.J. Koch) Hämet-Ahti	8.50	5.50	52.2	3	208

was added to the list in its place, as both its minimum and median flowering date fell between the first quartile and median of the flowering days for the species measured in the herbarium (Fig. 4). Following this change, the final candidate list was edited to include *Anthyllis vulneraria*, *Astragalus alpinus*, *Lathyrus japonicus*, *Lathyrus palustris*, *Lathyrus pratensis*, *Vicia cracca*, and *Vicia sepium.*

The large-scale compilation of data required for botanical databases presents challenges in data validation and verification. Data quality assessment is often overlooked in biodiversity database curation, as the process is time-consuming, particularly for levels of data validation that can only be done by experts in the field (Dalcin et al. 2012). The importance of data cleaning practices in biodiversity databases was highlighted in reports commissioned by the Global Biodiversity Information Facility, one of the largest biodiversity data infrastructures in the world (Chapman 2005a, b). The guidelines set out in these reports provide a standardized way in which to detect and address errors in biological collection databases. As the level of data validation for individual databases is often unknown to users, it becomes important to cross-reference any extracted data to ensure its accuracy. When utilizing these resources for selection of wild species, expert knowledge of the region or taxonomic group can provide an additional step of verification to confirm selection traits are accurately documented.

3.3 Seed collection

Between two and five populations were selected per species for seed collection to ensure that enough seed could be collected for the planned greenhouse and field experiments to study the agronomic potential of each species (Table 2). All populations selected were within a 150 km radius of Umeå, as seed maturation needed to be monitored frequently. Of the 44 populations identified in the region, 26 were selected for seed collection. Additional populations were initially identified in Artportalen, but when visiting the documented locations, no population was found. This discrepancy occurred

Table 2 Site collection, seed collection date range, and 1000 seed weight of each accession collected for the seven candidate species. Seed collection dates are from the year 2020.

Species	Latitude (decimal degrees)	Longitude (decimal degrees)	Seed collection date range	1000 seed weight (g)
Anthyllis vulneraria L. subsp. *lapponica* (Hyl.) Jalas	64.520298	18.830967	20/8-31/8	3.25
Anthyllis vulneraria L. subsp. *lapponica* (Hyl.) Jalas	63.834709	20.2552014	21/8-31/8	2.75
Anthyllis vulneraria L. subsp. *lapponica* (Hyl.) Jalas	64.668722	19.26149	10/8-5/9	3.62
Astragalus alpinus L. subsp. *alpinus*	64.520523	18.829605	20/8-31/8	1.01
Astragalus alpinus L. subsp. *alpinus*	64.442566	19.208264	10/8-5/9	1.11
Astragalus alpinus L. subsp. *alpinus*	64.505009	19.253225	10/8-5/9	1.02
Astragalus alpinus L. subsp. *alpinus*	64.669345	19.261334	10/8-5/9	1.34
Lathyrus japonicus Willd. subsp. *maritimus* (L.) P.W. Ball	63.782568	20.526322	22/8-10/9	32.53
Lathyrus japonicus Willd. subsp. *maritimus* (L.) P.W. Ball	63.654965	20.291008	30/8-10/9	37.78
Lathyrus palustris L.	63.782999	20.526056	22/8-10/9	7.76
Lathyrus palustris L.	63.656533	20.291636	30/8-10/9	7.35
Lathyrus palustris L.	63.898657	19.890315	23/9	12.54
Lathyrus palustris L.	64.045761	19.916771	7/9-23/9	10.26
Lathyrus pratensis L.	63.8197	20.204121	29/8-30/8	10.14
Lathyrus pratensis L.	63.9206186	19.8183436	30/8-9/9	8.33
Lathyrus pratensis L.	63.9308665	19.2166639	11/8-9/9	9.19
Lathyrus pratensis L.	63.802251	20.306394	14/8-23/9	8.01
Lathyrus pratensis L.	63.8100472	20.2437201	24/8-9/9	9.54
Vicia cracca L.	63.819646	20.203785	29/8-30/8	9.86
Vicia cracca L.	63.9208527	19.815952	9/9-23/9	11.32
Vicia cracca L.	63.960008	19.88141	7/9-23/9	12.92
Vicia cracca L.	63.7940575	20.5761457	22/9	11.29
Vicia sepium L. subsp. *montana* (W.D.J. Koch) Hämet-Ahti	63.793359	20.575594	22/9-10/10	19.65
Vicia sepium L. subsp. *montana* (W.D.J. Koch) Hämet-Ahti	63.920503	19.800377	11/8-23/9	14.09
Vicia sepium L. subsp. *montana* (W.D.J. Koch) Hämet-Ahti	63.5641364	19.4847991	11/8-23/9	16.79
Vicia sepium L. subsp. *montana* (W.D.J. Koch) Hämet-Ahti	63.931047	19.215614	11/8-9/9	18.32

most frequently with potential populations of *Vicia sepium*. In nearly all incidences of missing *V. sepium* populations, populations of *Vicia cracca* were found in its place. This population disparity is likely due to either misidentification of *V. cracca* for *V. sepium*, population decline between the date of record in Artportalen and the date of visitation, or incorrect spatial data in the database.

Seeds were collected between August 10th and October 10th, 2020, with *Anthyllis vulneraria* having the shortest duration of collection time (11 days) and *Vicia sepium* having the longest (61 days) (Table 2). Collection date was determined by seed pod maturity, which was defined as when seed pods turned brown to black and were near dehiscence. One thousand seed weights varied between accessions for each species. The smallest 1000 seed weight was *Astragalus alpinus* (mean, 1.12 g; standard deviation, 0.13 g), and the largest was *Lathyrus japonicus* (mean, 35.16 g; standard deviation, 2.63 g) (Table 2). The collection of seed is important not only for cultivation of these wild species to study their agronomic potential, but also to conserve their genetic resources through preservation in a gene bank. The inclusion of seeds from these wild legume species can contribute to the effort to conserve genetic diversity of crop wild relatives (Cowling et al. 2017; Fitzgerald et al. 2019).

3.4 Future development of selected species

The selection of these seven wild forage legume species is only the first step in a long process to potential cultivation and inclusion in leys. Much is still unknown about the selected species, and as such, extensive study of their agronomic potential is necessary. Understanding characteristics such as hard seededness, soil-type suitability, forage quality, anti-nutritional factors, and response to varying management intensities will be imperative in determining if these wild species can be included in cropping systems. Additionally, it is essential to identify their natural rhizobial symbiont, as commercial inoculants will need to be assessed for their suitability or new inoculants will need to be produced. Some work has been done to identify rhizobia of Swedish legumes through molecular methods, with *Anthyllis vulneraria*, *Astragalus alpinus*, *Vicia cracca*, and *Vicia sepium* already characterized (Ampomah and Huss-Danell 2011, 2016; Ampomah et al. 2017). Perhaps the most important factor to consider will be their potential for seed production. In order for these wild legumes to have a positive impact on the sustainability of ley production, they must first be capable of producing seed on a large enough scale to make their inclusion in leys economically viable (Boelt et al. 2015). Without commercial seed production, these species have no hope of being incorporated into leys on any meaningful scale.

Following the collection of seed from wild populations, additional work is being done to assess the agronomic potential of the seven wild, forage legume species selected. Germination studies, greenhouse experiments, field trials, pollination surveys, and nitrogen fixation analyses are in progress or already completed using the collected seed. The results from this work will help to further narrow down the list of candidate species and focus domestication efforts on the species with the most potential for inclusion in northern Swedish leys.

4 Conclusions

Here, we have shown that the use of botanical resources allows for the empirical selection of native forage legume species based on specified characteristics of interest. Though the use of herbaria and databases to consolidate data on plant species is not new, the effort to use this data to focus on targeted agronomic traits of interest for selection of wild species to include in a specific agricultural system is novel. Botanical databases provided a time efficient way to sort through key plant traits for selection. Regional floras gave local context to the extracted data, as characteristics of a single species can vary greatly over a geographic scale. Herbarium specimens contributed information on the morphology and phenology of local plant populations growing under similar climatic conditions to agricultural production in the region. Using these resources, seven wild forage legume species native to northern Sweden were chosen due to their potential for inclusion in leys. The utilization of botanical resources as a method of wild species selection for domestication offers an information-rich platform from which previously unconsidered species can be assessed for their agricultural potential.

Additional agronomic traits, such as persistence, rhizobia specificity, and soil-type suitability, could have provided supplementary selection criteria; however, these data were either unstudied or unavailable in database form. The utilization of constructed databases allows this method of selection to be a time efficient way to identify wild species with agricultural potential. Challenges arose during data acquisition but could be resolved through acknowledging potential biases in herbarium specimens and ensuring proper data validation when utilizing botanical databases. Increasing collaboration between agronomists interested in wild species and botanists focusing on economically important plant taxa could assist in ensuring that botanical data is accurately utilized during the selection process. Although applied to an entire taxonomic group in this study, these methods have potential to further narrow down existing crop wild relative inventories by agronomic traits of interest. Additional work to obtain data on the agronomic traits of the seven selected wild, forage legume species is underway and will provide new information on important characteristics such as forage quality, potential anti-nutritional factors, response to management, and persistence when grown in a grassland agricultural system.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval Not applicable

Consent to participate Not applicable

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Conflict of interest The authors declare no competing interests.

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Supplementary Material Table 1: The 79 legume species recorded in Sweden from the Swedish Virtual Herbarium. The selection criteria life cycle, habit, native range, and number of specimens are listed for each species. Recorded ethnobotanical uses are also included. Species with an asterisk are invalid.

Species	**Life Cycle**	**Habit**	**Status in Sweden**	**Number of Specimens**	**Ethnobotanical Uses**
Anthyllis vulneraria L.	Perennial	Non-climbing herb	Native	125	Forage, Medicine
Astragalus alpinus L.	Perennial	Non-climbing herb	Native	264	
Astragalus arenarius L.	Perennial	Non-climbing herb	Native	3	
Astragalus frigidus (L.) A. Gray	Perennial	Non-climbing herb	Native	34	
Astragalus glycyphyllos L.	Perennial	Non-climbing herb	Native	10	Forage, Medicine
Astragalus norvegicus	Perennial	Non-climbing herb	Native	35	
Astragalus oroboides Hornem.*				1	
Astragalus penduliflorus Lam.	Perennial	Non-climbing herb	Native	1	
Caragana arborescens Lam.	Perennial	Non-climbing tree	Introduced	24	Fibre, Food, Forage, Medicine
Cicer arietinum L.	Annual	Non-climbing herb	Introduced	2	Food, Forage, Medicine
Cytisus purpureus Scop.	Perennial	Non-climbing shrub	Introduced	1	
Cytisus scoparius (L.) Link	Perennial	Non-climbing shrub	Native	2	Fibre, Food, Forage, Medicine
Galega orientalis Lam.	Perennial	Non-climbing herb	Introduced	2	Forage
Genista pilosa L.	Perennial	Non-climbing shrub	Native	17	
Genista tinctoria L.	Perennial	Non-climbing shrub	Nativ	4	Fibre, Medicines
Laburnum x watereri (A.C.Rosenthal & Bermann) Dippel	Perennial	Non-climbing tree	Introduced	1	
Lathyrus aphaca L.	Annual	Non-climbing herb	Introduced	2	Forage, Medicine
Lathyrus cicera L.	Annual	Non-climbing herb	Introduced	1	Food, Forage, Medicine
Lathyrus inconspicuus L.	Annual	Non-climbing herb	Introduced	1	Forage
Lathyrus japonicus Willd.	Perennial	Non-climbing herb	Native	146	Food, Forage, Medicine
Lathyrus latifolius L.	Perennial	Climbing herb	Introduced	6	Forage
Lathyrus linifolius (Reichard) Bassler	Perennial	Non-climbing herb	Native	19	Forage
Lathyrus maritimus Bigelow*				1	
Lathyrus odoratus L.	Annual	Climbing herb	Introduced	2	
Lathyrus palustris L.	Perennial	Non-climbing herb	Native	163	Forage
Lathyrus pratensis L.	Perennial	Climbing herb	Native		Forage, Medicine
Lathyrus sylvestris L.	Perennial	Climbing herb	Native		Forage, Medicine
Lathyrus tuberosus L.	Perennial	Climbing herb	Introduced	3	Food, Forage, Medicine
Lathyrus vernus L. (Bernh.)	Perennial	Non-climbing herb	Native	7	Forage, Medicine
Lens culinaris Medik.	Perennial	Non-climbing herb	Introduced	2	Food, Forage
Lotus corniculatus L.	Perennial	Non-climbing shrub	Nativ	105	Food, Forage
Lotus tenuis Waldst. & Kit. ex Willd.*				2	
Lupinus angustifolius L.	Annual	Non-climbing herb	Introduced	2	Food, Forage, Medicine
Lupinus luteus L.	Annual	Non-climbing herb	Introduced	1	Forage
Lupinus nootkatensis Donn ex Sims	Perennial	Non-climbing herb	Introduced	16	
Lupinus polyphyllus Lindl.	Perennial	Non-climbing herb	Introduced	7	Forage
Medicago arabica (L.) Huds.	Annual	Non-climbing herb	Introduced	1	Forage

(Continued)

Species	e Cycle	Habit	Status in Sweden	Number of Specimens	Ethnobotanical Uses
Medicago falcata L.	Perennial	Non-climbing herb	Native	18	Forage
Medicago lupulina L.	Perennial	Non-climbing herb	Native	61	Forage
Medicago orbicularis (L.) Bartal.	Annual	Non-climbing herb	Introduced	2	Forage
Medicago polymorpha L.	Perennial	Non-climbing herb	Introduced	1	Food, Forage, Medicine
Medicago rigidula (L.) All.	Annual	Non-climbing herb	Introduced	1	Forage
Medicago sativa L.	Perennial	Non-climbing herb	Introduced	38	Food, Forage, Medicine
Melilotus albus Medik.	Perennial	Non-climbing herb	Introduced	57	Forage, Medicine
Melilotus altissimus Thuill.	Perennial	Non-climbing herb	Native	5	Forage, Medicine
Melilotus indicus (L.) All.	Annual	Non-climbing herb	Introduced	2	Forage, Medicine
Melilotus officinalis (L.) Lam.	Perennial	Non-climbing herb	Introduced	62	Food, Forage, Medicine
Onobrychis viciifolia Scop.	Perennial	Non-climbing herb	Introduced	1	Forage
Ononis spinosa L.	Perennial	Non-climbing herb	Native	16	Food, Medicine
Ornithopus perpusillus L.	Annual	Non-climbing herb	Native	4	Forage
Oxytropis lapponica (Wahlenb.) Gay	Perennial	Non-climbing herb	Native	39	Forage
Phaseolus vulgaris L.	Annual	Non-climbing herb	Introduced	6	Food, Forage, Medicine
Pisum sativum L.	Perennial	Climbing herb	Introduced	16	Food, Forage, Medicine
Robinia pseudoacacia L.	Perennial	Non-climbing tree	Introduced	1	Food, Forage, Medicine
Thermopsis montana Nutt.	Perennial	Non-climbing herb	Introduced	13	
Trifolium arvense L.	Annual	Non-climbing herb	Native		Forage, Medicine
Trifolium aureum Pollich	Perennial	Non-climbing herb	Native	22	Medicine
Trifolium campestre Schreb.	Annual	Non-climbing herb	Native	5	Forage
Trifolium dubium Sibth.	Annual	Non-climbing herb	Native	8	Forage
Trifolium fragiferum L.	Perennial	Non-climbing herb	Native	2	Forage, Medicine
Trifolium hybridum L.	Perennial	Non-climbing herb	Introduced	81	Forage
Trifolium incarnatum L.	Annual	Non-climbing herb	Native	6	Forage
Trifolium medium L.	Perennial	Non-climbing herb	Native		Forage, Medicine
Trifolium montanum L.	Perennial	Non-climbing herb	Native	1	Forage, Medicine
Trifolium pratense L.	Perennial	Non-climbing herb	Native	57	Food, Forage, Medicine
Trifolium repens L.	Perennial	Non-climbing herb	Native	84	Food, Forage, Medicine
Trifolium spadiceum L.	Perennial	climbing herb	Native		Forage, Medicine
Trigonella caerulea (L.) Ser.	Annual	Non-climbing herb	Introduced	1	Food, Forage, Medicine
Trigonella foenum-graecum L.	Annual	Non-climbing herb	Introduced	1	Food, Forage, Medicine
Vicia angustifolia L.*				1	
Vicia cracca L.	Perennial	Climbing herb	Native	138	Food, Forage, Medicine
Vicia faba L.	Annual	Non-climbing herb	Introduced	1	Food, Forage, Medicine
Vicia hirsuta (L.) Gray	Perennial	Climbing herb	Native	32	Food, Forage, Medicine
Vicia lathyroides L.	Annual	Non-climbing herb	Native	1	
Vicia sativa L.	Perennial	Non-climbing herb	Native	38	Food, Forage, Medicine
Vicia sepium L.	Perennial	Non-climbing herb	Native	96	Food, Forage
Vicia sylvatica L.	Perennial	Climbing herb	Native		Forage, Medicine
Vicia tetrasperma (L.) Schreb.	Perennial	Non-climbing herb	Native	10	Forage
Vicia villosa Roth	Perennial	Non-climbing herb	Introduced	6	Forage, Medicine