Protein digestibility of biorefined juice from fresh and ensiled ley crops in growing pigs

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Protein smältbarhet av bioraffinerad juice from färsk och ensilerad vall till växande grisar

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Abstract

The study aimed to evaluate the protein and amino acid digestibility of press juice from biorefined ley crops in the form of fresh (FPJ) and silage (SPJ) when included as an ingredient in liquid diets for pigs. The juice was extruded from ley crops mixture (grass-clover). The juice was stored in 10 L plastic containers and frozen at -20° C until the day of use. Eight female pigs (YH) around 9 weeks of age and an average of 24.15 (±2.76) kg BW were randomly assigned to one of the two dietary treatments containing FPJ (n = 4) or SPJ (n = 4). The diets were formulated to meet the nutritional requirements of the pigs, based on 4% of the average BW with the inclusion of 50 % of press juice in the mixture on a dry matter (DM) basis. The dietary treatments were mixed with a protein-free basal diet with an indigestible marker of titanium dioxide (TiO_2) in order to calculate the protein and amino acid digestibility. The experimental period lasted for eleven days with seven days for adaptation to the diet and four days of faecal sampling. At the end of the trial the animals were euthanized, and the ileal content was collected for analyses of crude protein, amino acids, and concentration of TiO_2. The differentiation of the indigestible marker obtain in the samples were used to calculate the apparent total tract digestibility (ATTD), apparent ileal digestibility (AID) and standardized ileal digestibility (SID) values of crude protein and amino acids for press juice in both fresh and silage form. A statistical general linear model (PROC GLM) was used to analyze the digestibility values between the two dietary treatments. The results of the trial showed that there was no significant difference ($p>0.05$) for gaining weight in pigs fed with FPJ or SPJ diets. The ATTD of crude protein (CP) and organic matter (OM) were higher ($p<0.05$) for the SPJ diet and the values for AID of CP, lysine, and threonine were unaffected by treatment, however, the values for AID of methionine were higher ($p<0.05$) for the SPJ diet. The SID of lysine and methionine was higher ($p<0.05$) for SPJ than FPJ diet, and despite the fact that SID of CP and threonine were higher for SPJ there was not significant difference ($p>0.05$). In conclusion, the acceptable levels of protein and amino acid digestibility of both FPJ and SPJ evaluated in growing pigs opens the potential to use the protein content found in these biorefinery fractions as an additional protein source to be considered in the inclusion of liquid diets for pigs.

Keywords: protein digestibility; amino acids; ley crops; silage; biorefinery; press juice; growing pigs

Table of contents

List of tables **7**

List of figures **8**

Abbreviations **9**

1. Introduction **10**
1.1 Aim and Hypothesis of the Study 11

2. Literature review **12**
2.1 Green Biorefinery 12
2.2 Feed value of biomass, press juice and cake from ley crops 13
  2.2.1 Protein and Amino acid profile 17
  2.2.2 Aspects of dietary protein in pigs 18
2.3 Digestibility 19
  2.3.1 Faecal and ileal digestibility 20
  2.3.2 Ileal digestibility and endogenous losses 21
2.4 Feeding aspects and liquid feeding system in pigs 22

3. Material and Methods **24**
3.1 Animal Ethics 24
3.2 Experimental forages and press juice handling 24
3.3 Animals and housing 25
3.4 Experimental design and treatments 25
3.5 Data collection 27
  3.5.1 Body weight and feed sampling 27
  3.5.2 Blood samples 27
  3.5.3 Faecal and Ileal sampling 27
  3.5.4 Sample analyses 28
  3.5.5 Calculations 28
  3.5.6 Statistical analyses 29

4. Results **30**
4.1 Chemical composition of the different fractions 30
4.1 Weight control 31
4.2 Apparent total tract, apparent ileal and standardized ileal digestibility 31

5. Discussion **34**

6. Conclusions 39

References 40

Popular science summary 46

Acknowledgements 48

List of tables

Table 1. Composition of fresh biomass, press-cake, and press-juice from grass-clover forage according to Santamaria-Fernandez et al.,(2019). 15

Table 2. Chemical composition (g kg^{-1} DM) of silage juice from ley crops mixture: timothy (P. pratense), meadow fescue (F. pratensis), red clover (T. pratense), and white clover (T. repens) in two different studies. (Keto et al., 2021; Presto Åkerfeldt et al., 2022) 16

Table 3. Amino acid composition (g kg^{-1} DM) from protein concentrates and silage juice from different green biomass (Taken from: Santamaria-Fernandez et al., 2019; Ravindran et al., 2021; Keto et al., 2021). 17

Table 4. Grass-legumes mixture in organic grassland in Sötåsen, Töreboda. 24

Table 5. Analyzed chemical composition and ingredients in the Basal feed diet (g/kg DM). 26

Table 6 Chemical composition of the experimental diets (g/kg DM). 26

Table 7 Chemical composition of the different fractions of biomass as fresh and ensiled (%DM) and amino acid (g kg^{-1}DM), (Fresh press juice, fresh press cake, silage press juice and silage press cake) 30

Table 8. Initial, final, and gained weight of the pigs (kg) under FPJ and SPJ diets (LSMeans and SE) 31

Table 9. Apparent total tract digestibility (ATTD), and apparent ileal digestibility (AID) [%] of crude protein, lysine, methionine, and threonine in FPJ and SPJ diets (least square means + standard error). 32

Table 10. Standardized ileal digestibility (SID) [%] of crude protein, lysine, methionine, and threonine in FPJ and SPJ diets (least square means + standard error) and their corresponding digestible amount in FPJ and SPJ diets (g/kg DM). 32

Table 11. Crude protein (CP) and Lysine provided by FPJ and SPJ diets (g/kg DM) based on the SID requirements(g/MJ NE daily) for growing pigs. 33

Table 12. Comparison of digestibility values between the present study with SBM (González-Vega et al., 2011) and WWDS (Pedersen & Lindberg, 2010) [%]. 37

List of figures

Figure 1. Schematic Process of Green Bio-refining and utilization of green biomass from fresh harvested or ensiled ley crops. (Modified by Perez Davila from Xiu & Shahbazi, 2015). 13

Figure 2. Green bio-refinery extruder plant at Sötåsen Naturbruksgymnasium i Töreboda-Sweden. 14

Abbreviations

AA	Amino acids
AID	Apparent ileal digestibility
ATTD	Apparent total tract digestibility
BCAA	Branched-chain amino acids
BW	Body weight
CP	Crude protein
DGW	Daily gain weight
DM	Dry matter
EAA	Essential amino acids
FCR	Feed conversion ratio
FPC	Fresh press-cake
FPJ	Fresh press-juice
GB	Green biomass
GBR	Green bio-refinery
OM	Organic matter
PC	Press-cake
PJ	Press-juice
SID	Standardized ileal digestibility
SPC	Silage press-cake
SPJ	Silage press-juice
TID	True ileal digestibility

1. Introduction

The search for sustainable feed sources with low environmental impact and locally produced crops is a constant challenge in pig production. During the last years the biorefining of green biomass of ley crops (grass and legumes) have gained special interest because of the different valuable products that are retained from the process and can be used as biofuel and feed to farm animals (Hermansen et al., 2017; Corona et al., 2018). This opportunity to use the perennial grassland becomes even more important if we consider that 44% of Swedish arable land (Swedish Board of Agriculture, 2021) are ley crops and in Europe, around 31.2% of the total agricultural area (Eurostat, 2020) are permanent grasslands.

Furthermore, the benefits of grasslands are not based only on the output of biomass nitrogen being higher than the annual monoculture crops but also because it provides a better N balance with less nitrate leaching to the soil (Aronsson et al., 2007; Manevski et al., 2018), resulting in better soil fertility and sustainable agriculture. Green biorefinery produces, after an extruding process, a fiber rich cake and a green juice with soluble nutrients such as sugars, proteins, free amino acids, dyes, and other substances (Kamm & Kamm, 2004). Platforms of biorefineries can allow the production of bioethanol but also the extraction of protein that could be used in animal feeding (Parajuli et al., 2015) in a circular bioeconomy system.

The yield of biomass from perennial grasslands or ley crops in green biorefinery could produce an important supply of protein and amino acids to substitute the imports of other sources of protein for feeding pigs in Europe. Studies of protein extraction from the green juice in biorefinery to obtain a dry protein concentrate, have shown positive potential to replace soybean meal in pig diets as a sustainable protein source for monogastric intake (Stødkilde, 2019; Ravindran et al., 2021). However, the protein extraction from the green juice involves procedures like centrifugation, precipitation, and drying process to obtain a protein concentrate (Xiu & Shahbazi, 2015). An alternative option is to include the juice fraction directly in the diet of the animal as an ingredient of the liquid feeding system of pigs (Adler et al., 2018). By conservation of the fresh ley crops into silage, a supply of the pressed juice could be obtained all-year-round. Silage juice can be a sustainable ingredient in the fattening of pigs when mixing with cereal-based feed at 2.6 to 4.1 lt/pig/day thus inclusion levels of 400g DM/day, 12 % of dietary energy and 23 % of the CP intake (Rinne et al., 2018) without affecting the feed efficiency or gut microbiota (Keto et al., 2021). Similar studies using silage juice from green biorefinery in liquid feeding of growing pigs showed no negative effects on growth or health at inclusion levels of 10 % from the dietary CP (Presto Åkerfeldt et al., 2022), however it is important to consider that silage nutrient composition could show a variation and thus will reflect on the silage juice extracted.

Concentration levels of CP between 190-265 g kg^{-1} DM in the liquid fraction of ensiled ley crops with 10 to 13% DM can be expected, depending on the variety of proportion of legumes and grasses, the age and wilting at harvesting, and the type of methodology used in the process (Franco et al., 2018). Silage juice with 10.8 % DM from ley crops with a mixture of timothy *(Phleum pratense*), meadow fescue (*Festuca pratensis*), English ryegrass (*Lolium perenne*), red clover (*Trifolium pratense*) and white clover (*Trifolium repens*) contained 157.4 g kg^{-1} DM of CP and 6.9 g kg^{-1} DM of lysine in a trial made by Presto Åkerfeldt et al., (2022) in Sötåsen, Töreboda, Sweden.

The liquid fractions of both fresh and ensiled ley crops after biorefining can be implemented in liquid feeding systems to pigs, considering that the quality of those fractions meet the nutritive requirements for pig feeding. However, to get a correct feed optimization, it is essential to know the nutrient digestibility for pigs found in both fresh and ensiled pressed juice from ley crops.

1.1 Aim and Hypothesis of the Study

This study aimed to evaluate the protein and amino acid digestibility of the pressed juice from biorefined fresh and ensiled ley crops in an in vivo pig trial to evaluate the potential to include the juice fractions as an ingredient in liquid diets to pigs.

This study hypothesized that press-juice from biorefined ley crops, as freshly harvested or ensiled, are highly digestible and therefore they can be used as an alternative protein source ingredients in the wet feeding diet for pigs.

2. Literature review

2.1 Green Biorefinery

In the last decades, the concept of green biorefinery (GBR) has taken great importance not only for the opportunity to produce renewable energy as an alternative to fossil fuels but also for the potential to obtain sub-products from this industry as feed ingredients in the animal feeding systems. Basically, green biorefineries use green biomass from grass and legumes as raw material to produce different fractions for different use e.g., the liquid fraction (press juice) containing proteins, amino acids, sugars, and dyes, and the fiber rich fraction (press cake) (Kamm et al., 2010).

Using grasslands as green biomass, not only represents a sustainable resource in the circular economy but also an environmentally friendly way to produce energy and protein supply to monogastric animals (Santamaria-Fernandez et al., 2018). The higher soil carbon bounding and the better N retention with less N leaching in the fields of grasslands compared to annual crops, can play an important role in soil conservation and sustainable agriculture (Manevski et al., 2018).

The schematic process applied in green biorefineries is shown in *Figure 1.* The process is relevant both using green biomass from ley crops (grass-clover) as fresh and as a form of silage. After the first step of fractioning, the green biomass is isolated in two economic value components, the fiber-rich press-cake and the nutrient-rich press-juice, from which a variety of products can be obtained at the end of the processing. Despite the different DM content in the juice and the cake, both can be used as biofuel substrates and feeding sources for animals. In this case, the fibrous cake as a dietary component in ruminants feed (Savonen et al., 2020) or processing the juice by separation, concentration, and drying to obtain protein concentrates to be used in monogastric animal feeding (Xiu & Shahbazi, 2015; Hermansen et al., 2017).

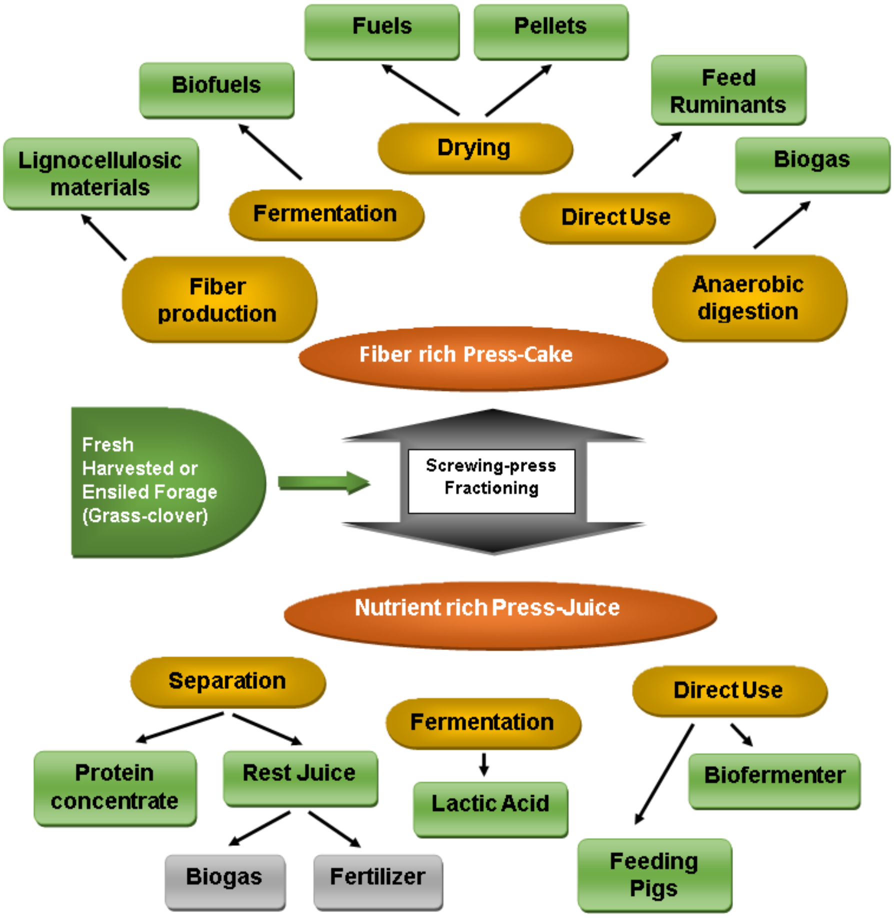

Figure 1. Schematic Process of Green Bio-refining and utilization of green biomass from fresh harvested or ensiled ley crops. (Modified by Perez Davila from Xiu & Shahbazi, 2015).

2.2 Feed value of biomass, press juice and cake from ley crops

The feeding value of green biomass from ley crops used as raw material for GBR involves certain considerations due to the high variability of the ley crops used and the conditions under which it was managed, such as the proportion of grass and legumes in the field, type of fertilization, age of harvesting, season of the year, weather conditions, and the level of wilting before processing, which will affect the nutritive value in the press juice and cake (Franco et al., 2018). The common way to extract the green juice from the green biomass is the screw press machine (*Figure 2*), obtaining a liquid fraction rich in proteins, free amino acids, enzymes, minerals, and other substances with great importance to nutrition and substrates for biogas production (Kamm et al., 2009).

Figure 2. Green bio-refinery extruder plant at Sötåsen Naturbruksgymnasium i Töreboda-Sweden.

However, the technology used in refining can influence the yield of the fractions at the end of the process. When the biorefining focuses on protein extraction, the separation process of the biomass can show a retention in the range of 50 to 70% of the DM with 40 to 60% of the protein in *the press cake fraction*, and a precipitated and separated wet protein paste containing 10 to 20% of the DM with 30 to 60% of the protein recovered, also known as *protein precipitation fraction* (Hermansen et al., 2017) this in theory can reach a value of 28% DM with 47% CP. Based on this information, if the yield of protein precipitation fraction is then set to a drying process, easily the range of the amount of protein can reach the one found in the soybean meal (50-55% CP DM) which could be replaced in monogastric dietary feeding while the fiber fraction still could be used in ruminant feeding, especially because the press-cake contains insoluble components like lignin, cellulose, hemicellulose, and fiber bound proteins (Corona et al., 2018).

Fresh biomass using a grass-clover mixture (*Table 1*) with 19% DM showed 29.9% and 8% DM for the fractions of press cake and juice respectively and a CP level of 17.2% in the green biomass resulted in 15.8% and 23.8% of CP DM in the press cake and juice respectively (Santamaria-Fernandez et al., 2019). This demonstrates that higher extraction of protein is obtained in the liquid fraction. Furthermore, the soluble nutrients are washed out into the juice fraction, and as a consequence, the increase of fibres such as NDF, ADF, and ADL in the press cake is much higher than the grass-clover biomass. Also, certain minerals like potassium, phosphorus, and chlorine, found in the vacuoles of vegetal cells are washed out to the press juice increasing the ash content (Santamaria-Fernandez et al., 2019).

Certainly, higher levels of potassium may cause loose faeces in pigs and the levels should not exceed the recommended 10g/kg diet (NRC, 2005).

Table 1. Composition of fresh biomass, press-cake, and press-juice from grass-clover forage according to Santamaria-Fernandez et al.,(2019).

Component	Fresh Biomass	Fresh Press-Cake	Fresh Press-Juice
Dry matter (%)	19.0	29.9	8.0
Crude Protein(%DM)	17.2	15.8	23.8
Ash(%DM)	10.0	8.1	15.7
NDF(%DM)	35.9	46.2	-
ADF(%DM)	19.1	25.2	-
ADL(%DM)	2.8	4.0	-

The extractable protein from green biomass in GBR is influenced by the different plant species, with forage feed quality higher in legumes than grasses, but also a decline in extracted protein is found with the increase of maturity in the ley crops, thus the best alternative for protein yield extraction in GBR will be legumes with harvesting at an early stage (Solati et al., 2017). To obtain good quality fodder proteins, the press juice must follow a series of processes including separation, precipitation, coagulation, heating, and drying, but also the use of biodegradable polymer-polylactide (PLA) method is applied to the supernatant protein through an anaerobic fermentation to produce L-lysine-L-Lactate (Xiu & Shahbazi, 2015).

Cong & Termansen (2016), found not only that substitution of cereal-based pig feed in diets with rich protein products from the GBR is an interesting idea from an economic perspective for the pig industry and GBR platforms, but also net benefits from an environmental perspective. Press cake can be used as food for ruminants in form of pellets or silage, but also from an energetic perspective, can be used as solid fuel or substrate for synthetic biofuels (Xiu & Shahbazi, 2015). The use of press juice as a direct component in pig feeding has been considered in the pig industry as an alternative way where a liquid feeding system is used. Studies have shown no difference in feed conversion ratio between pigs feed with diets including press juice and without juice. Press juice from pastures containing 80% timothy and meadow fescue, and 20% red clover, were used to feed pigs at the fattening stage in Norway, with the inclusion of 10% in the diet, and found no difference in feed conversion rate to control treatment but the ratio of omega-3: omega-6 in the pig’s fat were higher in those pigs fed with forage juice. This could be, due to high content of omega-3 fatty acid alpha-linolenic acid found in forages which could represent an additional benefit aspect, for human nutrition, the use of forage juice in feeding pigs (Adler et al., 2018).

To have a continuous and more efficient supply of press juice throughout the whole year, the green biomass can be ensiled (Kamm et al., 2016). Pig feeding trials in Finland have found that silage juice from mixed timothy (*P. pratense*) and meadow fescue (*F. pratensis*) green biomass, had great potential in liquid feeding for fattening pigs, without affecting daily growth rate, feed performance, or gut health (Keto et al., 2021). In that study 3 L of silage juice (7% DM with 27.9% CP) / pig and day was fed. The experimental diets corresponded to an inclusion ratio of 9.6 % of the dietary DM (Keto et al., 2021). However, Keto and colleagues (2021), recommend monitoring the use of the silage juice composition in pig feeding, due to the variability of silage quality, that can affect the energy intake and production output of the pigs. In Sweden, the growth performance of pigs was tested in a feeding trial using silage juice from ley crops mixture of grass-legumes: timothy (*P. pratense),* meadow fescue (*F. pratensis*), red clover (*T. pratense),* and white clover (*T. repens*). Silage press juice was added manually to a commercial feed to obtain a wet feed mixture, and no difference in growth was found between treatments with or without the inclusion of silage press juice (Presto Åkerfeldt et al., 2022). *Table 2* shows the chemical composition (g kg^{-1} DM) of silage juice from ley crops mixture used in the pig studies made in Norway and Sweden. According to Keto et al. (2021) and Presto Åkerfeldt et al. (2022), silage juice could theoretically replace 10 % of the CP content in liquid diets and thus represent a potential alternative as a local feed ingredient to pigs.

Table 2. Chemical composition (g kg^{-1} DM) of silage juice from ley crops mixture: timothy (P. pratense), meadow fescue (F. pratensis), red clover (T. pratense), and white clover (T. repens) in two different studies. (Keto et al., 2021; Presto Åkerfeldt et al., 2022)

	(Presto Åkerfeldt et al., 2022)		(Keto et al., 2021)	
	Silage biomass	Silage juice	Silage biomass	Silage juice
DM, %	32.0	10.8	21.0	7.0
Metabolizable energy, MJ kg^{-1} DM	11.4	10.0	-	-
Crude protein (g kg^{-1} DM)	127.0	157.4	144.0	279.0
Fat (g kg^{-1} DM)	32.0	37.0	-	3.4
Crude fibre (g kg^{-1} DM)	217.0	-	-	-
NDF (g kg^{-1} DM)	409.0	138.9	589.0	-
Ash (g kg^{-1} DM)	71.0	120.4	69.0	-
Lysine (g kg^{-1} DM)	-	6.9	-	13.6
Methionine (g kg^{-1} DM)	-	2.4	-	4.8
Threonine (g kg^{-1} DM)	-	5.6	-	10.9
Calcium (g kg^{-1} DM)	6.4	7.3	-	7.73
Phosphorous (g kg^{-1} DM)	2.7	9.5	-	8.81
Sodium (g kg^{-1} DM)	0.6	1.0	-	-
Potassium (g kg^{-1} DM)	23.1	36.5	-	70.7

2.2.1 Protein and Amino acid profile

The chemical analysis of *green protein concentrates* in comparison to green silage juice is shown in *Table 3.* In the study made in Ireland by Ravindran et al. (2021) the protein concentrate was obtained after biorefining and heating coagulation process of the green juice from freshly harvested perennial ryegrass. This protein concentrate held 40-50% of the total biomass soluble protein, together with sugars, minerals, and other nutrients while the rest of the protein remained in the press cake. However, the amino acid composition from this concentrate protein was slightly lower than in a study made in Denmark by Santamaria-Fernandez et al. (2019) where the protein concentrate was obtained after lactic acid fermentation of a green juice with 8% DM and 23.8% CP, biorefined from a grass-clover ley crop (45:55 ratio) made in Denmark.

Table 3. Amino acid composition (g kg^{-1} DM) from protein concentrates and silage juice from different green biomass (Taken from: Santamaria-Fernandez et al., 2019; Ravindran et al., 2021; Keto et al., 2021).

Amino Acid	Protein concentrate from Grass-clover *(Santamaria-Fernandez et al., 2019)*	Protein Concentrate from perennial rye grass *(Ravindran et al.,2021)*	Green silage juice from timothy and meadow fescue *(Keto et al., 2021)*
DM %	94.5	90.0	7.0
Crude Protein %DM	33.5	33.9	27.9
True Protein %DM	29.4	-	-
Crude Fiber %DM	-	6.1	-
Ash %DM	10.6	11.8	-
Alanine	23.4	21.2	21.6
Arginine	20.0	18.4	10.5
Aspartic	33.1	30.9	25.7
Cystine	2.3	1.8	0.4
Glutamine	35.1	35.8	30.3
Glycine	18.0	17.9	12.2
Histidine	7.5	6.5	2.4
Isoleucine	16.8	14.8	11.0
Leucine	27.8	27.5	19.0
Lysine	20.7	18.1	13.6
Methionine	6.3	6.5	4.8
Phenylalanine	18.1	18.4	10.3
Proline	14.3	15.2	12.2
Serine	14.4	13.8	10.6
Threonine	15.0	15.0	10.9
Tryptophan	-	6.1	-
Tyrosine	-	9.9	5.2
Valine	20.9	18.7	19.7

Moreover, a study made in Finland by Keto and colleagues (2021), found that the crude protein from silage juice of mixed grasses timothy (*P. pratense*) and meadow fescue (*F. pratensis*) had 279g kg^{-1}DM and a representative number of amino acids (*Table 3*), probably due to the lactic fermentation of silage (Keto et al., 2021). In fact, the idea of developing organic amino acids is not new, Kamm and colleagues (2009), described the production of lactic acid or lysine through basic techniques, including fermentation of the PJ carbohydrates to recover 90% of lactic acid from the sodium lactate fermentation broth and by ultrafiltration, 50% of lysine hydrochloride from lysine fermentation.

2.2.2 Aspects of dietary protein in pigs

The growth of pigs has intrinsic relation with the synthesis of proteins where the anabolic process interacts to build muscle through the protein turnover, however, it produces muscle waste if the catabolic process breakdown proteins that already exist in the animal (Norton & Layman, 2016). Dietary protein digestion begins in the stomach under the action of proteases and HCl. The catabolism of proteins is made by the action of a proteolytic enzyme (pepsin) into peptides and the successive breakdown of amino acids (AA) and oligopeptides by pancreatic protease in the small intestine (McDonald et al., 2002). The dietary protein degraded in smaller peptides and free amino acids (AA), can be used again in new protein synthesis or for further degradation in metabolites to generate energy as ATPs.

Amino acids are considered the building blocks in the synthesis of proteins and constitute the precursors in the different pathways of biosynthesis. The starting point is the amino acid synthesis and transcription of DNA into mRNA to be used in translation, all together with the substantial ribosomal content that makes pigs very efficient in protein synthesis at an early age (Yin et al., 2013a; Ren et al 2013).

The combination of 20 AA based upon a genetic code is responsible to constitute several thousands of proteins in the body. Those that cannot be synthesized by the body are called essential AA and must be included in the diet of pigs (Arginine, lysine, leucine, isoleucine, methionine, histidine, phenylalanine, threonine, tryptophan, and valine). The group of non-essential AA is not required to include in the diet because they can be synthesized by the body, using the process called transamination where a new AA is created when an amino group from one AA is transferred to an organic acid and catalyzed by transaminases enzymes (Cheek & Dierenfeld, 2010). Both essential and non-essential AA from dietary protein is degraded in the small intestine and are used (< 20%) for protein synthesis in the intestinal mucosa, using glutamine as the main energy source for the intestinal epithelium (Cheek & Dierenfeld, 2010; Yao et al., 2012) with great importance in the nutrition and health of the pigs. Apparently, AA are indirectly a source of energy in the postprandial stage, playing the role of precursors for neoglucogenesis and ketogenesis (Ren et al., 2013).

The digestion and metabolism of dietary protein and AA in pigs must be considered seriously in order to formulate the right diets and decrease the nitrogen excretion in the production (Zhang et al., 2012). Some factors can affect AA metabolism in pigs, and the gut microbiota is one of them, due to the capacity to hydrolyze and deaminate essential AA (EAA) in the intestinal lumen resulting in degradation and loss of EAA including branched-chain amino acids (BCAA), instead of being used for the synthesis of body protein (He et al., 2013). Protein deposition in growing pigs is the best parameter for growth and is considering that average daily gain (ADG) correlates with the net portal absorption of EAA (He et al., 2013). The importance of protein quality is restricted to the AA profile contained in the dietary protein that matches the AA requirements of the pig and meeting exactly those requirements will result in the optimization of nitrogen use and growth, also known as ideal protein (Cheek & Dierenfeld, 2010). High rates of insoluble fiber, concentration of glucosinolates, gossypol, and tannins, are not only the antinutritional factors that affects bioavailability of amino acids and reduce the protein digestibility in the most common feed protein products (soybeans and grain legumes), also trypsin inhibitors and Maillard reaction, after heat treatments (>140° – 165° C), will result in the reduction of available lysine (Sarwar Gilani et al., 2012). This problem affects monogastric animals, considering that lysine is the first limiting amino acid for pigs. However, all these inconvenience does not occur when using press juice as protein source in pig feeding, because no fiber content is found in the juice neither heat treatment is applied to it.

Although exist minor compounds found in plants at the swards with nutraceutical properties (vitamins & antioxidants) of important value also is possible to find cannabinoids, saponins and phytoestrogens, that are less studied, with unwanted biological presence during the biorefining process (Hermansen et al., 2017). It is known that in pastures with high proportion of legumes the concentration of phytoestrogens are higher, and because those are similar to the β-estradiol, they can affect the reproductive activity and fertility of grazing animals when the levels reach 25mg/kg DM (Wyse et al., 2022). However, in biorefining process to obtain subproducts to supply animal feeding, the determination of all antinutritional factors are not well researched.

2.3 Digestibility

The estimated level in the digestion of a determined nutrient in the diet is known as digestibility. Digestion activity involves a set of steps to absorb the nutrients in small molecules from the ingested diet. Alloenzimatic digestion (ruminants) can differ from autoenzimatic digestion (non-ruminants) because of the different microbiota present in the gut (rumen and hindgut) that produce enzymes to contribute to the digestion and absorption of nutrients (Cheeke & Dierenfeld, 2010). The majority of the digestion and absorption occurs in the small intestine parts, duodenum, jejunum, and ileum. The pancreatic activity releasing the proteolytic enzymes in the intestine to produce trypsin, chymotrypsin, and elastase are indispensable in the absorption of amino acids released from proteins and polypeptides (Cheeke & Dierenfeld, 2010).

Determination of digestibility can be made by in vitro or in vivo techniques with direct or indirect methods. Direct methods can be labour intensive, these include total tract collection which can be performed by using metabolic creates, measuring the input of nutrients in the pig diet, and the faecal output (McDonald et al., 2002). Indirect methods include the use of dietary inert markers where representative faecal samples or ileal digesta samples are taken for analyses and with the use of multiple regression being able to calculate digestibility values (Jagger et al., 1992).

Dietary inert markers most used in pigs are chromic oxide (Cr_2O_3) and titanium dioxide (TiO_2), however, despite the fact that Cr_2O_3 was the most used in digestible studies in pigs, TiO_2 has shown higher recovery rates than Cr_2O_3 (Jagger et al., 1992; Short et al., 1996), and is the most suitable today in digestible studies of N and AA in pig diets.

2.3.1 Apparent total tract and ileal digestibility

The most basic method to determine *in vivo* nutrient bioavailability is the apparent total tract digestibility (ATTD) but these faecal samples can show values including the enteric fermentation of undigested nutrients along the gastrointestinal tract (Columbus & De Lange, 2012). The digesta passing the hindgut of the pig, under the large influence of microbial metabolism, can be used as energy source. Microbial protein is synthesized by the traces of nitrogen from undigested and endogenous protein which alters the real values of the undigested dietary protein that enters the large intestine. This is the main reason why total tract digestibility alone could not have enough accuracy to determine protein and amino acid digestibility (Moughan, 2003).

However, the analysis of the digesta content at the end of the small intestine (ileum), could represent the undigested amounts of amino acids from the dietary protein (ileal digesta), considering that digestion and absorption are almost complete at this stage at the end of the ileum. Those values corresponding to the unabsorbed dietary amino acids could show a more reliable approach to calculate the protein and amino acid digestibility coefficient and is known as apparent ileal digestibility (AID)(Columbus & De Lange, 2012; Moughan & Miner, 2013).

In order to determine the values of AID, it is necessary to obtain samples of the digesta from the ileum of the pig. The techniques include different T-canulation techniques for the animal but also the slaughter technique which has been largely studied and compared, offering no significative difference in digestibility values between them (Zhang et al., 2013).

2.3.2 Endogenous losses, true and standardized digestibility

Despite the fact that ileal digestibility offers a better approach to amino acids digestibility than faecal digestibility, to avoid misleading, it must be considered a correction for amino acids from endogenous origin that are found in the undigested dietary amino acids to obtain a true ileal digestibility, otherwise will be only “Apparent” ileal digestibility (Moughan & Miner, 2013).

There are different approaches to calculate the ileal digestibility of AA. According to Stein et al., (2007), the calculation based on the different outflow of the ileal AA that is included can be expressed as apparent ileal digestibility (AID) if total ileal AA outflow is considered, and True ileal digestibility (TID) or standardized ileal digestibility (SID) if the endogenous losses are included.

When the content of AA and other nutrients is analyzed from the diet and ileal digesta or faecal samples, the calculation of apparent digestibility (AD) can be determined. Sauer et al., (2000) mention the equations applied to calculate AD for total collection, very similar to AID from Stein et al., (2007) (Eq. 1), but also mention AD for the indicator technique (Eq. 2) when an undigestible marker is used in the assay diet.

$$AD = [(AA\ diet - AA\ ileal\ or\ faeces)/\ AA\ diet] \times 100 \qquad \text{(Eq.1)}$$

$$AID = 100 - [(Marker\ diet \times AA\ ileal\ or\ faeces)/(Marker\ ileal\ or\ faeces \times AA\ diet)] \qquad \text{(Eq.2)}$$

In the ileal digesta is possible to find unabsorbed AA from the diet (exogenous origin) but also AA from microbial protein, secreted digestive enzymes, and protein from mucosal cells (endogenous origin) which form the ileal endogenous AA losses. These endogenous losses could be basal endogenous or specific endogenous losses (Stein et al., 2007). Basal endogenous losses are nutrients that animals lose influenced by the total DM intake more than the ingredient diet they are fed with. However, specific endogenous losses are the losses related to the innate characteristics of the feed ingredient like anti-nutritional aspects or fiber content and could be estimated by removing the basal endogenous losses from the total endogenous losses (Stein et al., 2007).

Sauer et al., (2000) consider the true ileal AA digestibility (TID) value based on both the AD and the amount of endogenous AA found in the ileal or faecal digesta (Eq. 3).

$$TID = AD + (AA\ endogenous\ ileal\ or\ faeces\ /\ AA\ diet) \times 100 \qquad \text{(Eq. 3)}$$

Meanwhile, Stein et al., (2007) consider the true ileal AA digestibility as the amount of dietary AA that is not found in the distal ileum and without considering ileal endogenous AA losses either (Eq. 4). However, TID values must be avoided in diet formulation due to the lack of accuracy in determining the total ileal endogenous losses.

$$TID = [(AA\ diet-(Ileal\ AA\ outflow-Total\ ileal\ endogenous\ AA))/AA\ diet] \times 100 \qquad \text{(Eq. 4)}$$

Finally, standardized ileal digestibility (SID) is calculated as the AID considering the withdrawal of the basal endogenous losses (Eq. 5) either by using protein-free assay diets, high digestible protein sources, or by regression method. (Stein et al., 2007).

$$SID= [(AA\ diet-(Ileal\ AA\ outflow-Basal\ ileal\ endogenous\ losses))/AA\ diet] \times 100 \qquad \text{(Eq. 5)}$$

One of the major advantages of using SID is that only the basal endogenous losses are taken out from ileal digesta leaving the rest of the endogenous values restricted to the feed ingredient to be part of the calculation which could be helpful in diet formulation.

2.4 Feeding aspects and liquid feeding system in pigs

Pigs need a reasonable amount of nutrients to cover the most basic physiological aspects but also require investing energy to grow, gain weight, and other metabolic processes related to reproduction, milk synthesis, or thermoregulation. The cost of feeding in pig production represents more than 60% and dietary energy states for 70% of that cost (Noblet & Van Milgen, 2004). The dietary energy comes from feed intake in the form of protein, carbohydrates, and lipids that after oxidation deliver a certain amount of gross energy (GE). For every gram of protein, carbohydrates, and lipids the GE delivered accounts for 5.6 kcal g^{-1}, 3.7 kcal g^{-1}, and 9.4 kcal g^{-1} respectively (McDonald et al., 2002).

Feeding pigs can be made in a variety of forms, liquid feeding is one of them, and despite the fact that has been used since the domestication of pigs still is today one of the most versatile ways to provide a well balance diet to the animals. Liquid pig feeding must have around 20 to 30 % DM and can be prepared from mixing water to dry food components or could be provided as fermented or non-fermented mixtures. Liquid feeding mixture can use liquid by-products from the dairy industry, brewing industry, starch industry, or even from the human food industry as well as bio-refinery sub-products.

Fermented liquid feeding has lower pH and improves the pig performance due to the reduction of pH level in the digestive tract which results in fewer pathogen bacteria proliferation (enterobacteria) avoiding gastrointestinal problems and improving intestinal morphology (Canibe & Jensen, 2003) and certainly, lower presence of coliforms could represent lower risk for gastrointestinal infections which means less antibiotics in the production cycle. Liquid feeding is the best way to achieve accurate feeding, increase dry matter intake and nutrient digestibility, increase DGW and FCR in fattening pigs (Missotten et al., 2015) and even reduce the dust in the pig barns.

To avoid a spontaneous fermenting, of the liquid feeding, a safe fermenting process with a series of inoculation techniques are suggested, and this seems to be not only safe but also a cost-effective option to maintain animal health and reduce the use of antibiotics in the pig industry (Plumed-Ferrer & von Wright, 2009; Missotten et al., 2015).

By using pressed silage juice instead of water when mixing with commercial feed, it was able to theoretically replace 10 % of the dietary protein content and at the same time obtain the same performance as the control group in gaining weight (Presto Åkerfeldt et al., 2022). This aspect shows the potential of pressed juice from ley crops as a local feed ingredient for organic or conventional pig farming.

3. Material and Methods

3.1 Financial support and animal Ethics

This experiment was carried out in September 2022 at the research facilities of the Swedish Veterinary Medicine and Animal Science Centre (VHC) in the Swedish University of Agricultural sciences (SLU) Uppsala, Sweden (59°48’54’’N, 17°39’23’’E). The project was financed by Stiftelsen Svensk Grisforskning (D-nr 2021-2). All animal procedures used during the trial were approved by the Research Animal Ethics Committee of Uppsala Region (SLU-ID: 5.8.18 - 03495/2021) in compliance with EC Directive 86/609/EEC regarding animal experiments.

3.2 Experimental forages and press juice handling

The juice was extruded from an organic grassland planted in 2021, located in Sötåsen Naturbruksgymnasium, Töreboda (Sweden). The grass-legumes mixture consisted of timothy (*Phleum pratense*), meadow fescue (*Festuca pratensis*), English ryegrass (*Lolium perenne*), red clover (*Trifolium pratense*), and white clover (*Trifolium repens*) *(Table 4).*

Table 4. Grass-legumes mixture in organic grassland in Sötåsen, Töreboda.

Type/Specie	Commercial name	%
Red clover *(Trifolium pratense)*	Vicky	2
Red clover *(Trifolium pratense)*	SW Ares EKO	8
White clover *(Trifolium repens)*	SW Hebe	4
White clover *(Trifolium repens)*	SW Hebe EKO	3
Timothy *(Phleum pratense)*	Switch	8
Timothy *(Phleum pratense)*	Switch EKO	32
Meadow fescue *(Festuca pratensis)*	SW Minto EKO	19
Meadow fescue *(Festuca pratensis)*	Tored	4
English ryegrass *(Lolium perenne)*	SW Birger	8
English ryegrass *(Lolium Perenne)*	SW Birger EKO	12

Harvesting was made in the summer season (July 14th) and to obtain the fresh press juice (FPJ), it was extruded (80% capacity) right away, the same day, without field wilting. The grass-legumes mixture aimed to be used for the silage press juice (SPJ) was, after harvest, treated with 0.5% formic acid (Promyr XR 580) and stored in silage pits. After the ensiling time of around 8 weeks, it was extruded under the same conditions (80% capacity) as the fresh one. Extruded FPJ and SPJ were stored in 10lt plastic containers and frozen at -20° C respectively until the day of use.

3.3 Animals and housing

Eight female pigs Yorkshire x Hampshire (YH) from around 9 weeks of age with average body weight (BW) of 24.15 ± 2.76 kg were transported from the Swedish Livestock Research Centre Lövsta to the VHC research facility (Department of Clinical Sciences - stable 3) at Ultuna, SLU. The pigs were housed individually in concrete floor pens (1.5 * 1.8 m) with solid wall sides and metal bars in front, equipped with water nipple drinkers and feed troughs. Also, they were supplied with individual heat lamps to keep a comfortable temperature. The pigs had an adaptation period of one week (period 1), where they had access to commercial feed for growing pigs which was mixed gradually with press juice from fresh (FPJ) and silage (SPJ) ley crops for three days to acclimatize and get used to the taste of the juice prior to the experimental period. In this period, it was allowed for the pigs to have access to straw as bedding material and nose contact with the neighboring pig. Before the experimental period (period 2) the pens were washed clean and rubber mats were placed in the pens as bedding on most of the pen floor. During this period straw was restricted to keep the animals free from ingestible sources other than the experimental diet. The pigs had visual contact with other pigs and had access to toys (balls and chains) as environmental enrichment of the pen to alleviate boredom. At the start of the experiment, underwent a health control by the resident veterinarian. The monitoring of the pigs for disease or injury and cleaning of the pens were done twice daily during the whole trial. Also, information from the animals like the initial and final weight was registered.

3.4 Experimental design and treatments

The pigs were randomly assigned to one of the two dietary treatments containing press juice from fresh (FPJ, n=4) or silage (SPJ, n=4) ley crops mixed with a protein-free basal diet with an indigestible marker of titanium dioxide (TiO_2) *(Table 5).* The diets were formulated to meet the nutritional requirements of the pigs, based on the 4% of the average BW of the animals at the start of the experiment with the inclusion of 50 % of either FPJ or SPJ in the mixture of dry matter (DM) basis *(table 6)*. The pigs were fed twice a day (morning and afternoon).

Table 5. Analyzed chemical composition and ingredients in the Basal feed diet (g/kg DM).

Description	Amount
Analyzed chemical composition g/kg DM,	
DM	954.5
Crude protein	0.0
Ash	25.1
NDF	41.7
TiO_2	2.17
Basal feed ingredients g/kg DM,	
Wheat starch	842.00
Dextrose	62.86
Cellulose	62.86
Rapeseed oil	41.91
Monocalcium phosphate	23.83
Salt	8.50
Premix	2.57
TiO_2	3.14

Table 6 Chemical composition of the experimental diets (g/kg DM).

	FPJ diet	SPJ diet
Ingredients (g/kg DM)		
Fresh press juice	500	
Silage press juice		500
Basal feed diet	500	500
Chemical composition (g/kg DM)		
DM	526.4	522.40
Crude protein	70.20	76.95
Ash	98.85	89.50
Cysteine + Cystine	0.66	-
Histidine	1.88	-
Isoleucine	0.37	2.38
Leucin	7.07	4.21
Lysine	5.19	3.66
Methionine	1.38	1.33
Phenylalanine	4.58	2.27
Threonine	4.22	2.88
Valine	4.83	3.32

FPJ= fresh press-juice, SPJ=silage press-juice

The experimental period lasted for 11 days with seven days for adaptation to the experimental diet and four days of faecal sampling. After four days of faecal sampling, the pigs were euthanized with a lethal injection in order to collect ileal digesta. Because both the pressed juice from fresh and silage were kept frozen at -20° C in 10 lt plastic containers, they had to be taken out of the freezer 36 hours before use to melt down completely. The mixing of both press juice and the basal diet had to be done by hand in buckets before being fed manually and individually to the pigs, in order to obtain a homogeneous mixture without clumps. Prior to each feeding event, eventual feed residuals from previous feeding occasion were noted (i.e., empty, half empty, or a lot left in the troughs), however not collected. And the feeding troughs and pen floors were then cleaned.

3.5 Data collection

3.5.1 Body weight and feed sampling

At the beginning of the experiment, the pigs were weighed to calculate the daily feeding allowance (based on 4% BW) to be offered in two times, at 7:00 and 15:00 h. Because the diet was based on 50% basal protein-free (95.45 %DM) containing TiO_2 (3,14 g kg $^{-1}$ DM) and 50% juice (9.83% DM and 9.03% DM for fresh and ensiled juice respectively), the amount of feed mixed per pig at the time of feeding was 2.5lt juice + 284 gr basal feed twice daily. The pigs were weighed on day 1 (initial weight: 24.15 ± 2.76) and on day 11 (final weight: 24.61 ± 2.66), which correspond to the day when the animals were euthanized.

Feed samples of green biomass, pressed juice, and the press cake from fresh and ensiled ley crops were collected *in situ* and immediately frozen (at -20° C) until the day of analysis for chemical composition.

3.5.2 Blood samples

Blood samples were taken from the jugular vein at the beginning and at the end of the study to check for the health status of the animals, and after centrifugation, serum, and plasma were frozen at -20° C until the day of the analysis.

3.5.3 Faecal and Ileal sampling

Faecal samples were representative collected from the floor twice daily at 7.00 and 15.00 h before feeding. These samples were weighed in a petri dish at approximately 25 gr/collection time for four days and were frozen at -20° C. For the sampling of ileal digesta, the pigs were euthanized and directly taken to autopsy to collect approximately 30 cm of the distal ileum which is identified by the proximity to the large intestine by the ileocecal valve. The ileal content was then emptied in a petri dish, weighed and immediately frozen (-20° C) before the time of the analysis.

3.5.4 Sample analyses

The chemical analyses of fresh and ensiled biomass, and their respective press juice and press cake, as well as in the faecal and ileal samples refer to DM, ash, crude protein (CP), amino acids content, organic matter (OM), and TiO_2.

Samples were freeze-dried before analysis. Chemical analysis for DM from ileal digesta, faecal, and feed samples including FPJ and SPJ was made by drying the samples at 103°C for 16 h and the analysis for ash content by ignition at 550°C for 3 h (Jennische & Larsson, 1990). To analyse the content of nitrogen (N) in the samples the method by Kjeldahl (Nordic Committee on Food Analysis 1976) was used. Then the N values were multiplied by the factor 6.25 to find the crude protein (CP) value for every sample. TiO_2 in feed, faecal and ileal digesta was analysed by the method described by Short et al., (1996) as a standard operating procedure. The absorbance was made at 405 nm using a microplate photometer reader (Thermo Scientific Multiskan FC^{TM}). The use of TiO_2 as an indigestible marker is important in order to be able to calculate the apparent ileal (AID) and total tract digestibility (ATTD) coefficients from FPJ and SPJ in pigs. Analysis of pH in FPJ and SPJ was directly made from the liquid fraction by a pH-meter (pH-meter, Metrohm 654. Schweitz). Amino acids were analysed according to EU 152/2009 ISO 13903:2005 (Eurofins) by hydrolysis of samples in HCL to break peptide bonds.

3.5.5 Calculations

With the chemical analyses information, the next step was to determine the apparent total tract digestibility (ATTD) for CP and OM; the apparent ileal digestibility (AID) and the standardized ileal digestibility (SID), for CP, lysine, methionine, and threonine for the FPJ and SPJ used as ingredients in the pig feeding by using the equations (Sauer et al.,2000; Stein et al., 2007):

$$\text{ATTD} = 1 - [(TiO_{2D} \times DC_F) / (DC_D \times TiO_{2F})],$$

where, ATTD is the coefficient of the apparent total tract digestibility of the dietary component (%), TiO_{2D} is the titanium dioxide concentration found in the treatment diet (g kg $^{-1}$ DM), DC_F is the dietary component found in the faeces (g kg $^{-1}$ DM), DC_D is the dietary component found in the treatment diet (g kg $^{-1}$ DM), $TiO_{2\,F}$ is the titanium dioxide concentration found in the faeces (g kg $^{-1}$ DM).

$$\text{AID} = 1 - [(TiO_{2D} \times DC_{Ig}) / (DC_D \times TiO_{2Ig})],$$

where, AID is the coefficient of the apparent ileal digestibility of the dietary component (%), TiO_{2D} is the titanium dioxide concentration found in the treatment diet (g kg^{-1} DM), DC_{Ig} is the dietary component found in the ileal digesta (g kg^{-1} DM), DC_D is the dietary component found in the treatment diet (g kg $^{-1}$ DM) and TiO_{2Ig} is the titanium dioxide concentration found in the ileal digesta (g kg $^{-1}$DM).

Calculations of the values for standardized ileal digestibility (SID) using the following equation:

$$\text{SID} = \text{AID} + (IL_E / DC_D) \times 100,$$

where, SID is coefficient of the standardized ileal digestibility of the dietary component (%), AID is the coefficient of apparent ileal digestibility of the dietary component (%), IL_E correspond to the ileal losses from endogenous N or AA (g kg $^{-1}$DM intake), and DC_D is the dietary component in the treatment diet (g kg^{-1} DM) (Stein et al., 2007; Høøk Presto et al., 2011). The mean values used for the IL_E [g kg^{-1} DM intake] corresponding to N and AA endogenous losses were taken from the study by Høøk Presto et al., (2010), based on pigs with an estimated 25,9 kg BW: CP= 8.625, Lys= 0.33, Met= 0.10 and Tre= 0.51 (Høøk Presto et al., 2010).

3.5.6 Statistical analyses

All data obtained were analysed using the GLM procedure of SAS, version 9.4 (SAS Institute Inc., 2021) using each animal as the experimental unit. Significant effects and differences between treatments in the pig growth, ATTD, AID, and SID of the CP and AA were made by including treatments (FPJ diet and SPJ diet) as fixed effects. The model was tested using least square means (t-tests) and presented as least square means with standard error (SEM), considering a statistical significance at $P \leq 0.05$.

4. Results

4.1 Chemical composition of the different fractions

Fresh and ensiled biomass analyses in *Table 7* show the CP, ash, NDF, pH, and AA content in the different fractions of pressed juice and pressed cake (pulp) after being extruded. CP content in FPJ (14.4 %DM) was lower than SPJ (15.39 %DM) although it came from the same harvesting field and at the same time. Chemical analyses of SPJ showed a lower pH compared to FPJ, but FPJ showed a slightly higher amino acids content than SPJ with exception of isoleucine.

Table 7 Chemical composition of the different fractions of biomass as fresh and ensiled (%DM) and amino acid (g kg^{-1}DM), (Fresh press juice, fresh press cake, silage press juice and silage press cake).

	Fresh Biomass	Ensiled Biomass	FPJ	SPJ	FPC	SPC
DM %	29.13	24.57	9.83	9.03	36.97	48.07
Crude Protein (% DM)	17.02	17.78	14.04	15.39	17.61	18.05
Ash (% DM)	8.83	7.95	17.26	20.43	7.65	5.62
NDF (% DM)	37.25	41.94	-	-	44.06	49.07
pH			5.40	4.19		
Alanine	10.17	10.67	11.70	9.85	10.64	10.61
Arginine	8.38	8.49	9.87	3.99	9.38	9.38
Aspartic	17.65	17.28	24.31	17.27	17.37	17.47
Cysteine+Cystin	1.14	1.12	1.32	-	1.15	1.19
Phenylalanine	8.31	9.22	9.16	4.54	9.20	9.93
Glutamine	16.79	17.71	20.45	13.62	18.01	18.87
Glycine	8.56	9.28	9.26	5.65	9.35	9.91
Histidine	3.27	3.56	3.76	-	3.59	3.90
Hydroxyproline	-	-	-	-	-	-
Isoleucine	6.26	7.29	7.30	4.76	7.05	7.59
Leucin	12.87	14.32	14.14	8.41	14.45	15.44
Lysine	9.05	8.84	10.38	7.31	9.77	9.68
Methionine	2.52	2.81	2.75	2.66	2.86	2.98
Ornithine	0.19	0.36	-	1.33	0.18	0.25
Proline	7.81	8.39	9.46	7.09	8.40	8.44
Serin	7.38	7.49	8.44	6.31	7.74	7.86
Threonine	7.78	7.84	8.44	5.76	7.93	8.32
Valin	8.17	9.33	9.66	6.64	9.20	9.70

4.2 Pig weights

The initial weight for the pigs in the FPJ diet did not differ from the SPJ diet ($p>0.05$) and because of the short period of the study (11 days), the gaining weight of the animals was not substantial either (*Table 8*). Therefore, the statistical analysis revealed that there was no difference ($p>0.05$) between the two treatments for final weight and gained weight. Blood sample analyses showed no abnormal values or health problems in the animals. During the experimental period there were observations of some feed refusals, and it was noted that it took place with more frequency in the afternoon and mostly by the smaller animals, however the observations for feed refusals in the morning where scarce and the animals were showing more appetite. As previously stated, the quantity of feed refusals were not collected but it was replaced with a new amount of food after troughs and pens had been cleaned.

Table 8. Initial, final, and gained weight of the pigs (kg) under FPJ and SPJ diets (LSMeans and SE).

	FPJ - diet	SPJ - diet	SE	*P*-value
Initial weight	23.60	24.70	1.458	0.613
Final weight	24.20	25.10	1.413	0.660
Gained weight	0.55	0.37	0.225	0.602

4.3 Apparent total tract, apparent ileal and standardized ileal digestibility

The data collected for the apparent total tract digestibility (ATTD) were based on a total of 8 pigs (n=4 pigs/diet), however, the apparent ileal digestibility (AID) and standardized ileal digestibility (SID) of crude protein were based on 6 animals (n=4 FPJ diet and n=2 SPJ diet). In the same way, the AID and SID of lysine, methionine, and threonine had only 7 observations (n=4 FPJ diet and n=3 SPJ diet), in both cases this missing value was due to that very little ileal content could be collected.

The ATTD of CP was higher in the SPJ diet than in the FPJ diet (70.8 vs 60.5%, $p<0.05$), however, the AID of CP showed no significant difference ($p=0.116$). The SPJ diet in the study had a higher ATTD for organic matter (OM) ($p=0.002$) than the FPJ diet. The AID for lysine tended to be significantly lower ($p=0.068$) in SPJ than in FPJ diet (*Table 9*). The SID of CP showed no significant difference ($p= 0.127$) *(Table 10)*, however the SID for lysine was significantly lower ($p=0.044$) in SPJ than FPJ diet. Both AID and SID for methionine showed higher ($p=0.014$ and

0.013) values for the SPJ than the FPJ diet. Meanwhile, the AID and SID for threonine were numerically but not significantly higher in SPJ than the FPJ diet *(Table 9 and 10).*

Table 9. Apparent total tract digestibility (ATTD), and apparent ileal digestibility (AID) [%] of crude protein, lysine, methionine, and threonine in FPJ and SPJ diets (least square means ± standard error).

	FPJ diet	SPJ diet	*P*-value
ATTD			
Crude protein	60.5 ± 2.42	70.8 ± 2.42	0.024
Organic matter	73.2 ± 1.24	82.7 ± 1.24	0.002
AID			
Crude protein	62.0 ± 3.99	75.8 ± 5.64	0.116
Lysine	71.3 ± 2.45	80.0 ± 2.83	0.068
Methionine	81.2 ± 1.68	90.7 ± 1.95	0.014
Threonine	75.6 ± 2.18	80.0 ± 2.51	0.251

Table 10. Standardized ileal digestibility (SID) [%] of crude protein, lysine, methionine, and threonine in FPJ and SPJ diets (least square means ± standard error) and their corresponding digestible amount in FPJ and SPJ diets (g/kg DM).

	SID (%)			SID (g/kgDM)	
	FPJ diet	SPJ diet	P-value	FPJ diet	SPJ diet
Crude protein	68.1 ± 3.99	81.4 ± 5.64	0.127	95.61	125.27
Lysine	74.5 ± 2.45	84.5 ± 2.83	0.044	7.73	6.18
Methionine	84.8 ± 1.68	94.5 ± 1.95	0.013	2.33	2.51
Threonine	81.7 ± 2.18	88.8 ± 2.51	0.085	6.90	5.11

Based on the SID values obtained for FPJ and SPJ diet in this study, the digestible amount (g/kgDM) of CP, Lysine, methionine, and threonine are shown in *Table 10.* The amount SID of CP (N x 6.25) and Lysine required for growing pigs in high production and maintenance level in relation to the proportion of these nutrients supplied by the FPJ and SPJ diet to the animals during this study are shown in *Table 11.*

Item	Requirement	Component in diets g/kg DM	SID g/kg DM in diet	SID supplied by diet %

	g/MJ NE day	g/d	FPJ	SPJ	FPJ	SPJ	FPJ	SPJ
SID CP	12.00[a]	148.8	70.20	76.95	47.81	62.64	32.13	42.09
SID Lys	1.03[a]	12.77	5.19	3.66	3.87	3.09	30.28	24.18
SID CP$_m$	(2.06g/kg BW$^{0.75}$) [b]	23.03	70.20	76.95	47.81	62.64	207.60	271.99
SID Lys$_m$	(0.071g/kg BW$^{0.75}$) [b]	0.79	5.19	3.66	3.87	3.09	489.87	391.14

Table 11. Crude protein (CP) and Lysine provided by FPJ and SPJ diets (g/kg DM) based on the SID requirements(g/MJ NE daily) for growing pigs.

(a)= Energy requirements based on 12.4 MJ NE/d for 25 kg for higher production growing pigs (Näringsrekommendationer för aminosyror till grisar, 2010).

(b)= Standardized ileal digestibility of CP (expressed by N x 6.25) and Lysine requirements for maintenance (NRC, 2012).

5. Discussion

The purpose of this study was to evaluate the protein and amino acid digestibility of pressed juice from the biorefined freshly and ensiled biomass of ley crops and evaluate the potential to be included as an ingredient in the liquid diets of pigs. As previous studies have shown, the chemical analysis of the different fractions of perennial grasslands can vary in nutrient content basically due to different factors such as the proportion of legumes or grasses, the age of wilting at harvesting, and the process used in the extruding (Solati et al., 2017; Franco et al., 2018) and that influence directly in lower or higher values. In this study, the amount of protein and amino acids found in the chemical analysis of the juice fractions were much lower than other studies made by Santamaria-Fernandez et al., (2019), Ravindran et al., (2021) and Keto et al., (2021). Despite the fact that crude protein in the fresh and ensiled biomass were acceptable, the slightly higher retention of crude protein in the press cake means that during the extraction process, some aspects like the harvesting, the swards management, the type of species in the pasture or even the extruding process itself may have affected the yield of the extractable protein content per kg DM; which has been stated before by Hermansen et al., 2017; Franco et al., 2018, and in consequence less protein solubles were yielded in the pressed juice from both fresh and ensiled biomass despite the fact that the extruding technique was equal repeated in both cases.

The digestibility study was conducted in vivo and by following an adapting time before the experimental period and during this process the animals were healthy from the beginning to the end of the trial. The low increment of weight in the animals during the short period of the experiment (n= 11 days) was probably because the experimental diets had a limited nutritive content as we can see in the chemical analysis of the diets *(Table 6).* A closer overview of this situation is explained in the *Table 11,* showing that the protein requirements the animals need for commercial production were only partly cover by the FPJ and SPJ diets about 32% and 42% respectively. The same situation was for lysine requirements and only 30% and 24% was covered by FPJ and SPJ respectively. Therefore, it seems like in this case the FPJ and SPJ diets were covering more than double of the maintenance for crude protein requirements and almost 4 times the lysine requirements for maintenance (2.06g/kg BW$^{0.75}$ Sid CP, 0.071g/kg BW$^{0.75}$ Sid Lys (NRC, 2012)) but not the necessary lysine for protein deposition and the same applies for other amino acids (Van Milgen et al., 2008; Van Milgen & Dourmad, 2015). In trials where semi-purified diets with a sole source of protein (in this case from FPJ or SPJ) as an ingredient of interest is evaluated, it could result in imbalanced amino acid profile and impaired condition for the pig to maintain the optimal physiological condition, resulting in low weight gain (Park et al., 2018). Therefore, mixing the pressed juice with a protein-free basal diet was intended to perform a digestibility study of the ingredient of interest and not for the purpose of measure the gaining weight as in standard production. However, the inclusion of press juice together with other feed ingredients in nutritional balanced pig for growing and fattening pigs has been tested in several studies (Adler et al., 2018; Rinne et al., 2018; Keto et al., 2021; Presto Åkerfeldt et al., 2022). These studies have shown that replacing part of protein from other feed sources with pressed juice in pig diets are possible without affecting the productive performance of the animals.

It is necessary to point out the absence of previous protein or amino acid digestibility studies in pigs using fresh or ensiled pressed juice from ley crops, so there are no previous coefficients of digestibility to compare the ones observed in the present study. However, studies made by Stødkilde et al., (2019) with DM, amino acids, and N digestibility of protein concentrates from different fractions of red clover, white clover, lucerne, and perennial ryegrass in lab rats, found 85% of N digestibility from lucerne as one of the best protein concentrates of these green plants. Stødkilde and colleagues (2019), also found that a significant amount of soluble protein can be extruded in the biorefining process and after precipitation could be used in monogastric feeding with high values of digestibility (Stødkilde et al., 2019). In the present study, the ATTD of CP was found 60.5% and 70.8% for FPJ and SPJ respectively, which is a substantial digestibility value to take into consideration that ley crops (grass-legumes mixture) juice fraction protein is as good as those from highly digestible protein sources (Sarwar Gilani et al., 2012). However, when comparing FPJ vs SPJ effect in weight increment in the pigs, it was not significant, but it is interesting to observe better digestibility values of ATTD of crude protein (60.5% vs 70.8%) and organic matter (73.2% vs 82.7%) from FPJ compared to SPJ respectively. As it was mentioned before the data collected for the apparent total tract digestibility (ATTD) were based on a total of 8 pigs, 4 on each diet and the apparent ileal digestibility (AID) and standardized ileal digestibility (SID) of crude protein were based on n=4 and n=2 for FPJ and SPJ diet respectively. However, 7 observations corresponded to the AID and SID of lysine, methionine, and threonine, n=4 and n=3 for FPJ and SPJ diet respectively. Considerations regarding the sample size of the study is necessary to remark, because the number of animals involved in the experiment was not large and in addition some missing values occurs because very little ileal content was collected. This have likely contributed to the lack of significant differences between FPJ and SPJ in for example the AID of CP and lysine although the numerically differences are rather large and have probably a biological impact (13.8 and 8.7 % differences, respectively).

It is important to remark that while freshly harvested leys are extruded right away to get FPJ, the SPJ is obtained from the silage after a minimum 2 months of anaerobic fermentation where the homo fermentative bacteria digest the soluble carbohydrates of the biomass into acetic and lactic acid and lowering the pH. It has been demonstrated that feeding pigs with low pH content can be beneficial for the animals because enhance the gut microbiota lowering the pathogen bacteria and promoting beneficial bacteria (Canibe & Jensen, 2003). In addition, lower pH in the stomach and high lactic acid concentrations enhance proteolytic activity in the pig digestive system and may therefore improve digestibility (Missotten et al., 2015).

In order to obtain an optimized growth and reduce costs and N excretion in the pig production, it is necessary to know the true ileal values of CP and AA digestibility (Yin et al., 2013b). It is necessary not only to get the AID, that could give us a general value of digestibility according to the availability of the nutrients in the ileum, but also the SID which takes into perspective the basal and the specific endogenous ileal AA losses in the pig digestion (Libao-Mercado et al., 2006). SID is considered as the true ileal digestibility (NRC, 1998) and help to quantify the metabolic cost of the animal in the AA digestion.

The AID of CP and AA (lysine, methionine, and threonine) observed in the present study did not differ between FPJ and SPJ, except for the AID of methionine, which was higher for SPJ than FPJ (90.7% vs 81.2%). The SID of CP and AA (lysine, methionine, and threonine) were, on the other hand, higher for lysine (84.5% vs 74.5%) and methionine (94.5% vs 84.8%) in SPJ compared to FPJ. All these values of digestibility observed in the present study show that nutrients in juice from fresh and ensiled grass-legumes mixed are digestible by the pigs and that these fractions have the potential to supply important protein and AA compounds in the pig diets. The values of protein and AA digestibility from FPJ and SPJ are in the range of some commonly used ingredients in pig diets. Compared to soybean meal (SBM) used in the study by Gonzales-Vega et al., (2011), FPJ show lower digestibility values of all compared amino acids and SPJ slightly lower for all compared amino acids except methionine and threonine which show similar AID and SID values as SBM (*Table 12*). In the same way, wheat wet distillery soluble (WWDS) digestibility values found by Pedersen & Lindberg (2010), could be compared to FPJ and SPJ and it is shown that crude protein and lysine digestibility of FPJ and WWDS are rather similar whereas for the other compared amino acids and for SPJ higher values than WWDS are reported (*Table 12*). The results found in the present study support the previous positive results obtained in the studies where juice fractions from ley crops have been used in pig diets without affecting pig performance or meat quality (Adler et al., 2018; Keto et al., 2021; Presto Åkerfeldt et al., 2022).

Table 12. Comparison of digestibility values between the present study with SBM (González-Vega et al., 2011) and WWDS (Pedersen & Lindberg, 2010) [%].

	SBM *(González-Vega et al., 2011)*	WWDS *(Pedersen & Lindberg, 2010)*	Present Study	
			FPJ diet	SPJ diet
AID				
Crude protein	84.6	61.0	62.0	75.8
Lysine	90.5	64.0	71.3	80.0
Methionine	90.5	58.0	81.2	90.7
Threonine	83.1	51.0	75.6	80.0
SID				
Crude protein	93.1	75.0	68.1	81.4
Lysine	93.0	75.0	74.5	84.5
Methionine	93.2	66.0	84.8	94.5
Threonine	89.2	63.0	81.7	88.8

SBM= soybean meal (not heated), WWDS= wheat wet distillery solubles.
FPJ= fresh-pressed juice, SPJ= Silage-pressed juice.
AID= apparent ileal digestibility, SID= standardized ileal digestibility.

Based on the results of digestibility values for crude protein and some of the most important AA in this study, it is possible to approximate the amount of AA expressed in SID g/kg DM that these liquid fractions (FPJ and SPJ) can provide when they are used as feed sources in wet feeding systems *(Table 9)*, and in this way contributing to more sustainable pig production in the region using local protein supply.

The values of SID of crude protein shows a biological relevance between FPJ and SPJ. It is reasonable to think that the sample size matters even in a small-scale study, however, in this case both FPJ and SPJ shows acceptable digestibility values and depending on the availability, they can be used as ingredient in liquid feeding of pigs and cover part of the protein supplementation in the diet, in agreement with the previous studies made by Adler et al., (2018), and Presto Åkerfeldt et al., (2022). The benefits of using the fractions of FPJ and SPJ, after biorefinery, directly in the liquid feeding of pigs compared to a protein concentrate is that it may contribute economically not only because of it saves energy by avoiding unnecessary procedures involved to obtain the protein extraction (Xiu & Shahbazi, 2015) but also contributes to the better use of local resources and reducing long distance transport. The juice should be transported by using chilled trailers with temperatures under 4^{o} C. Furthermore, the green biorefinery on a small scale as part of a circular bio-economy have the potential to improve the soil carbon and N retention by using grass-legumes pastures as biomass (Manevski et al., 2018) to provide local protein feed resources to the pig industry in the most resilient way (Santamaria-Fernandez et al., 2018). In regions where the grasslands harvesting is made in specific months of the year, the use of fresh juice fraction needs to be monitored to avoid quick spoiling and must be put under a refrigeration system if it is not used immediately. However, the advantage of the biomass silage process presents a better convenient form of conserving and providing a continuous supply of pressed juice (silage biorefining) to the feeding of pigs all year round (Kamm et al., 2016; Keto et al., 2021). In order to guarantee an optimal output of the by-products in GBR, it is necessary to improve the quality of biorefinery focusing on the yield efficiency of the extruded juice and preserving the quality of the obtained protein from the fresh or ensiled green biomass (Stødkilde et al., 2019; Presto Åkerfeldt et al., 2022).

6. Conclusions

Fresh and ensiled extruded juice obtained from the green biorefinery process of grass-legume biomass used in this study showed acceptable protein and amino acid digestibility. They can therefore be interesting to use as ingredients in pig diets. The apparent and standardized ileal digestibility of lysine and methionine were higher for the ensiled press juice (SPJ) diet compared to the diet with fresh press juice (FPJ). In general terms both FPJ and SPJ could be included in liquid diets to pigs not only because of the acceptable digestibility of the protein and AA but also for the condition as a local liquid protein source. Further research in nutrient digestibility values of the liquid fractions of green biomasses in pigs is needed in order to establish standard coefficients of digestibility to be implemented when formulating pig rations.

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Popular science summary

The use of local protein supply obtained from grasslands not only represent a sustainable way of production and reducing the import of protein sources but also improve the ecosystem with better soil fertility, nitrogen and carbon bounding.

Green biorefinery use perennial grasslands to produce fuel but also a diverse variety of sub-products as part of the circular bio-economy. The extruding process of ley crops separate a rich fiber pulp and a press juice with interesting nutritive compounds to feed animals. The soluble protein, sugars, and other nutritive components found in the extruded juice can be used as an ingredient in the liquid feeding for pigs. An important question is how much of the protein and amino acids retained in the press juice that can be digested by growing pigs.

To develop this study, the juice from fresh and ensiled a mixture of grass-legumes pasture was extruded. These operational activities were made between July and September 2022, from an organic managed pasture located in Sötåsen Naturbruksgymnasium, Töreboda (Sweden).

For the animal trial 8 female pigs (YxH) 9 weeks of age with average body weight of 24.15 ± 2.76 kg were allocated in individual pens at the VHC research facility at SLU. The animals were assigned to receive for 11 days one of the two dietary treatments with press juice either from fresh or ensiled ley crops. The diet was a mixture of press juice mixed with a basal feed containing an indigestible marker, titanium dioxide (Ti_2O).

The apparent ileal digestibility of crude protein, lysine and threonine were not different for the diets with fresh or silage juice, but the apparent ileal digestibility of methionine was higher for the diet with silage juice. The standardized ileal digestibility of lysine and methionine were higher for the silage juice diet but did not differ for crude protein and threonine. The standardized protein digestibility was of 68 and 81% for fresh and silage juice respectively, which are acceptable and can validate previous research where press juice from green biorefinery have been fed to pigs with positive results.

Using juice from ensiled ley crops could, considering the conservation aspects, have the advantage of a more sustainable way to provide a continuous supply of juice.

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Publishing and archiving