BIOREFINERY
GLAS

BIOREFINERY GLAS

Final Report

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CARBERY

Grassa GREEN REFINED SOLUTIONS

UCD DUBLIN

Barryroe CO-OPERATIVE LIMITED

BIOREFINERY GLAS IS A EUROPEAN INNOVATION PARTNERSHIP (EIP) FUNDED BY THE DEPARTMENT OF AGRICULTURE, FOOD, AND THE MARINE (DAFM) UNDER THE RURAL DEVELOPMENT PROGRAMME 2014-2020

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1. EXECUTIVE SUMMARY

Biorefinery Glas is an EIP-Agri project which has taken place over a two-year period between February 2019 to February 2021. The focus of the project was to demonstrate and evaluate a small-scale grass biorefinery process with Irish farmers, particularly dairy farmers from the Carbery Group as well as pig farmers. The project successfully completed a biorefinery demonstration period over 6 weeks during the summer of 2019 producing product samples for analysis while demonstrating in practice the biorefinery unit in operation with the farmers. During the following months a range of products were tested and analysed by project partners, with favourable results. These included a presscake fibre containing a fraction of the grass protein which yielded comparable results on milk production compared with silage, while also demonstrating a lower emission of nitrogen, phosphorous and methane in dairy cows. The protein not included within the fibre was isolated as a separate co-product, and was trialled as a wet and dry protein additive for pigs, showing particular promise as a dry feed ingredients and achieving an increase in weight gain among pigs compared with a traditional weaner ration. From the residual whey stream of the refinery a prebiotic solution of fructo-oligosaccaharides was isolated and showed excellent early potential for development of a high value grass-based prebiotic ingredient for animal or human nutrition, while the final streams have also been evaluated, showing good promise in bio-fertiliser and bioenergy applications.

Following validation of the technology and products, and benchmarking of individual products, a business case was completed to understand the potential economic opportunity for Irish agriculture. This analysis shows a positive business case could be deliverable by a scenario in which 3 products (excluding fertiliser and energy) are considered, with the most profitable scenario being the production of 3 co-products (presscake, protein and fructo-oligosaccharides) alongside biogas production in a model which integrates biorefining and anaerobic digestion with CHP.

Overall, the project findings have been very positive and demonstrate the untapped potential of Irish grasslands to produced additional products to traditional dairy and meat products. It also demonstrates a model which could be integrated well at small-scale alongside and complementary to, traditional livestock farming. Given Irelands competitive advantage when it comes to growing grass, the developments and findings of Biorefinery Glas provides enormous opportunities for sustainable bioeconomy diversification and replication, in a model which supports the active participation of farmers in value creation, bringing significant economic, social and environmental benefits.

2. Brief Description of the Project

The Biorefinery Glas Project is a European Innovation Partnership (EIP) project funded by the Department of Agriculture Food and Marine as part of Ireland's Rural Development Programme 2014-2020. The Biorefinery Glas project is an Operational Group consisting of 5 project partners; Munster Technological University (formerly Institute of Technology Tralee) (co-ordinator), University College Dublin, Carbery Group, Barryroe Co-operative and GRASSA B.V. Work on the project commenced in February 2019 and ran until project completion at end of February 2022. Biorefinery Glas main project focus was to improve the sustainability, value and resource efficiency of Ireland's livestock sector through farmer diversification into the bioeconomy. The project demonstrated a replicable small-scale biorefinery with farmers in the West Cork Region. Through biorefining, perennial ryegrass was fractionated into a variety of new products in a process which improves the protein efficiency, value and sustainability of our grasslands. This report summarises the work undertaken by the project team during the 2-year project. The main achievement of the project included successful implementation of biorefinery demonstration with farmers in Cork to produce new products for testing, while improving farmer knowledge of the biorefinery and building awareness of the project. The project also successfully evaluated a range of new products from grass, which show significant potential for marketable diversification opportunities while providing environmental benefits. A successful business case analysis was also conducted.

2.1 Geographical Scope

In terms of geographical scope, the Biorefinery Glas project focused on the NUTS 3 South-West region and in particularly the west Cork area. The main focus areas were farms within the 4 west Cork co-operatives; Bandon, Barryroe, Drinagh and Lisavaird, participating as the Carbery Co-operative. The main participant farmers within the project were from from this area, however results were further disseminated among farmers and other stakeholders regionally, nationally and internationally.

Figure 1: Geographical Scope of the project

2.2 Background and Context for Project

In 2019, the Irish Government produced its "AgClimatise" Draft National Climate & Air Roadmap for the Agriculture Sector to 2030 and Beyond. The report highlights the latest EPA figures which suggest that agriculture accounts for 34% of national greenhouse gas emissions - 20.6 Mt CO2eq of a total 60.51

Mt CO2eq[1]. Of these emissions, methane is largest contributor (64.5%) followed by nitrous oxide (35%)[2]. The EPA projects that total national GHG emissions by 2030 will increase by 6% under the status quo. Meanwhile the 'All-of-Government Climate Action Plan to Tackle Climate Breakdown' places a target of 10-15% reduction in GHG emissions in agriculture by 2030. There is also a specific target to reduce ammonia levels by 5% by 2030 compared to 2005 levels (the agriculture sector accounts for 99.1% of ammonia emissions in Ireland). Reducing N-losses to air and water is another key priority for Irish agriculture. The urgent need to decarbonize the agriculture sector was reflected nationally in the 2017 Citizens assembly on Climate Action in which 89% of participants supported a tax on agricultural emissions[3].

Another challenge which Irish agriculture faces is around over-reliance on imported protein. This was highlighted by the fodder crises of 2013 and 2017-2018 which resulted in increasing feed prices. Excluding extreme events like the 2017-2018 fodder crisis, Ireland already imports over 3 million tonnes of protein feed annually, over half of which is GMO soy or maize and much of which has been sourced from South America, bringing a large carbon footprint due to its transport and impact on land-use and rainforest deforestation. The issue of protein imports is not only an Irish one. At European Level the EU recently announced their plans for launching an EU Protein Plan to address significant and long-standing feed protein deficits in the EU.

It is against this backdrop that the Biorefinery Glas project has been implemented, in order to show that bioeconomy can implemented at small-scale, providing farmers the opportunity to produce sustainable new products, while tacking key emissions challenges and protein shortage.

2.3 Biorefinery Glas Approach for resource efficient, low emission agri-sector

The Biorefinery Glas approach, as noted by "Ag-Climatise", along with the Biobased Industries Joint Undertaking[4] represents an effective way of improving farmer integration within bioeconomy value chains using a small-scale biorefinery approach. In this way, farmers can generate new revenue streams through diversifying their grasslands while addressing some of the challenges indicated above. As indicated in figure 2 below, during the biorefinery process, fresh grass is separated into a fibre press cake fraction and a juice fraction. The press-cake, containing only part of the protein, is fed back to the cow, where it can help to address key emissions challenges while still allowing the cow to deliver comparable milk output when compared with unrefined silage. The juice fraction is further processed to extract the remaining protein fraction which is isolated and can be fed to monogastrics such as pigs and poultry, replacing imported soybean and improving national protein resilience. A high value stream of sugars, in the form of fructo-oligosaccharides can be extracted and used as a prebiotic. Meanwhile, the residual stream, a nutrient-rich whey can be used as a fertiliser or in the production of renewable biogas energy through anaerobic digestion. Through the Biorefinery Glas approach, a significant

[1] Dept. of Agriculture (2019) 'Ag-Climatise' – A Draft National Climate & Air Roadmap for the Agriculture Sector to 2030 and Beyond
[2] Department of Communications, Climate Action and the Environment (2017) National Climate Mitigation Plan
[3] Citizens Assembly (2018) Third Report and Recommendations of the Citizens' Assembly – How the State can make Ireland a leader in tackling Climate Change
[4] Biobased Industries Joint Undertaking (2020) Study on participation of agricultural sector in the Biobased Industries Joint Undertaking.

reduction in N, P and CH4 emissions can be achieved in cattle while also greatly improving the availability of protein.

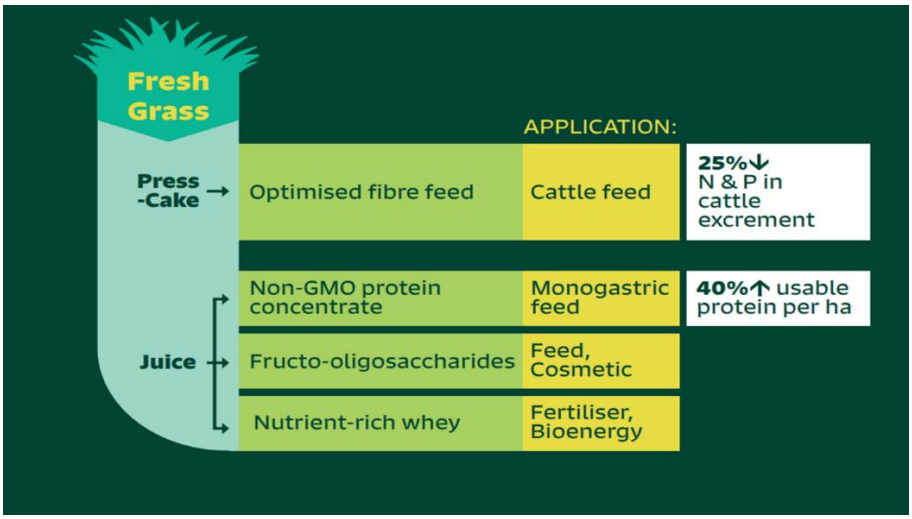

Figure 2: Biorefinery Glas Schematic from AgClimatise

To implement the project, Biorefinery Glas implemented 6 interlinked Work Packages with results flowing between the tasks. The Work Packages, leaders and contributors are outlined below.

WP1: Demonstration of Small-scale Grass Biorefinery (Lead: Grassa)

WP2: Validation of Products and Co-products Produced in the Biorefinery Process (Lead: UCD)

WP3: Business Case, Policy Development and Regulation (Lead: ITT/MTU)

WP4: Bioeconomy Knowledge Exchange Activities with Farmers (Lead: Carbery)

WP5: Dissemination for Irish farming community (Lead: ITT/MTU)

WP6: Project Management and Coordination (Lead: ITT/MTU)

3.0 PROJECT IMPLEMENTATION AND RESULTS

3.1. Onboarding of Farmers for Demonstration Activities

At the project outset a selection of participating farmers was made across the Carbery Co-operative. The participating farmers would host and facilitate biorefinery demonstrations on their farms, supporting the OG in the delivery of the project demonstration phase. The biorefinery was mobile and would be transferred between participating farms on a week to week basis. The purpose of the demonstrations was two-fold; on one hand to produce a sufficient quantity of the various biorefinery co-products streams in order to conduct product trials, while at the same time giving farmers first-hand experience of the biorefinery process and managing the system on their farm. In order to improve the visibility of the project farmers were selected across each of the west Cork Co-operatives of Bandon, Barryroe, Lisavaird and Drinagh, and centrally at Shinagh Estates Farm. Two pig farms were also selected, a dry pig feeding farm from Barryroe and wet pig feeding farm in Tipperary. All selected dairy farmers were members of Carberys Greener Dairy Farms Programme. In order to prepare for the biorefinery demonstration phase, selected farmers participated in a project onboarding programme, which introduced farmers to partners, as well as the technology and products. During the meetings, logistical issues around implementing the technology and related activities on farms were discussed and an implementation plan was put in place, detailing farm schedules, routes between farms, supporting infrastructure required for harvesting and transporting grass, storing products etc.

As part of onboarding activities, a Digital Storytelling Workshop was also hosted to familiarise participating farmers in the preparation of videos from their demonstrations. Videos were later captured and edited by farmers and submitted as dissemination materials under the project's Digital Storytelling Initiative. These videos are available to view on the project website.

Figure 3: Onboarding Meeting with Participating Farmers

Figure 4: Biorefinery Glas Digital Storytelling Workshop with Farmers

3.2 Biorefinery Demonstrations

Demonstrations of the biorefinery began on 1st July 2019. The biorefinery technology spent 1 week in full demonstration mode on each farm (2 weeks on Shinagh farm), before being transferred to the next farm over the weekend to commence a new demonstration. The biorefinery process operates at a max capacity of 2 tonnes fresh weight per hour, and during the product trials the aim was to operate at between 1 and 2 tonnes fresh weight per hour. Each day farmers ensured that grass was harvested using a zero grazer and transported to the machine. This was done twice daily in order to ensure fresh grass was available. An operator from project partner GRASSA was responsible for the day-to-day operation of the machine, with support from the participating farmers and the wider team. The grass was first transported to a loading bay in front of the refinery, and using a mini loader was transported into the loading dock for processing. During the process grass was washed and then crushed to remove some of the protein into a juice fraction with the fibre fraction containing the remaining protein being transported to the front of the machine via a conveyer. The fibre fraction (or presscake) was ensiled and baled at the end of each day to preserve for feeding trials. The bales were later transported to UCD Lyons Farm for use in dairy feeding trials (see section 3.3.1). Pallets and intermediate bulk containers we organised on each farm for collection of the grass juice and its co-product components. The grass juice was heated to concentrate the remaining protein which was extracted and stored in IBCs for use in pig feed trials. (see section 3.3.2) In the case of dry pig feed, a Dorset dryer was used to dry the feed up to 90% DM. The protein was then transported to Barryroe Co-op to prepare for use in pig feed trials. The residual whey was stored on IBC's on each farm. A part of this whey was transported to MTU to undergo a separation process for the extraction of fructo-oligosaccharides and a validation of their

prebiotic efficacy (see section 3.3.3). Part of the whey was also preserved for evaluation for use in the production of biogas/biomethane through anaerobic digestion (see section 3.3.5). Finally, the remaining material was spread on the farms of participating Carbery to evaluate its fertiliser potential on the plots from which the original processing grass was harvested (see section 3.3.4). (More information on all product testing and benchmarking in 3.3).

Figure 5: Transport of mobile grass biorefinery

Figure 6: Grass biorefinery in operation on farm with collection of presscake (front of machine) and whey in IBC

Figure 7: On farm activities during demonstration phase (i) harvesting grass (top left), (ii) baling presscake (bottom left) (iii) loading machine

3.3 Baseline Data

From the grass biorefinery, 4 co-processing streams were produced which were used within trials to benchmark against existing products and generate baseline data regarding the potential of the grass biorefinery value chain. The first is a high DM press-cake fibre feed which trialled as a cattle feeding source by UCD Lyons Farm. From the residual green juice fraction, the remaining protein was extracted into a protein concentrate, leaving a liquid stream called grass whey, rich in sugars and minerals. The protein was trialled as a wet feeding alternative pig feed ingredient on a commercial pig farm in Co. Tipperary, while a second batch of grass protein was dried and trialled by a commercial pig farm in Barryroe Co-op. At MTU, the residual biorefinery whey stream underwent a process for extracting and evaluating the prebiotic potential of sugars contained in the grass whey. While the remaining whey underwent further analysis to assess its biomethane and fertiliser potential. An overview of trial results is provided below.

Figure 8: Fresh green juice (left) and press cake (right) products of initial pre-processing

Figure 9: Products derived from fresh green juice; FOS liquid (left) Protein concentrate (middle), grass whey (right)

3.3.1 Press-cake cattle feed trial data

The analysis of press-cake fibre (PC) as an alternative feeding source to baseline product grass silage (GS) was conducted by UCD at Lyons Research Farm. The aim of the cattle feeding trial was to evaluate the effect of direct replacement of high-quality grass silage with biorefined press cake silage in the diet of dairy cows on cows' performance (daily intake; milk production and quality; rumen fermentation; nitrogen and phosphorus excretion). Analysis of the two feed materials is provided in Table 1 below, which shows that presscake contains lower crude protein compared with silage (this is expected due to protein being pressed out during the biorefinery process to produce a separate protein feed for pigs). The press cake also has lower NDF and ADF but higher DM.

Table 1: Experimental Feeds

Chemical composition (% of DM unless stated)	Experimental feeds			
	Grass silage	Press cake silage	Standard concentrate	Soya bean meal
DM (%)	29.94	37.41	90	88
Ash	9.83	4.20	6.92	8.66
Crude Protein	16.44	10.93	18.82	53.25
NDF	49.09	74.09	15.35	7.54
ADF	28.97	41.33	7.86	4.16
WSC	4.26	3.56	-	-
Starch	0.31	0.26	9.71	0.51
Phosphorus	0.42	0.39		
Ether extract	3.46	2.82	2.52	1.29
Gross energy (MJ/kg of DM)	17.65	18.30	17.70	19.60

Thirty early lactation autumn calving dairy cows were selected from the commercial herd in UCD Lyons Farm and the trial was conducted over a 10-week period (27/11/2019-06/02/2020), with a 14-day acclimatisation period and a 56-day experimental period. The cows were assigned to one of two dietary treatments as follows: **T1** (GS) consisted of grass silage (14kg DM) plus concentrate (7.4 kg DM) and soya bean meal (0.44 kg DM) and **T2** (PC) had the same quantities of concentrate and soya bean meal but 2/3 of the grass silage was replaced with press cake silage (5kg DM grass silage; 9 kg DM press cake silage) – an overview of the two treatments is provided in Tables 2 and 3 below.

Table 2 and 3. Two dietary treatments for Grass Silage (GS) and Presscake (PC), with silage replacement rate with PC of 66%

	GS	PC
Grass silage	14 kg DM	5 kg DM
Press cake silage	-	9 kg DM
Standard concentrate	7.2 kg DM	7.2 kg DM
Soya bean meal	0.44 kg DM	0.44 kg DM

(% of DM unless stated)	GS	PC
DM (%)	41.18	48.63
Ash	10.04	6.75
Crude Protein	18.02	15.22
NDF	37.33	56.94
ADF	22.52	27.07
WSC	4.61	2.25
Starch	3.55	4.42
Phosphorus	0.46	0.44
Ether extract	4.68	2.55
Gross energy (MJ/kg of DM)	17.98	18.15

The dietary treatments were offered ad libitum as a TMR via a diet feeder into individual feeders. Half of the concentrate was offered in the parlour at milking twice daily, while the remaining feed was offered in the TMR. Feed samples were collected every day for nutritional analysis. During the experimental period milk, rumen fluid and blood samples were taken once a week to assess respectively milk production and quality, rumen fermentation parameters (ammonia and volatile fatty acid concentration), metabolic status (glucose and beta-hydroxybutyrate). The impact of presscake dietary treatment on milk yield and composition is provide in Table 4 below. Despite significantly less crude protein in the presscake, the analysis shows **no significant difference in milk yield or quality compared** with the cows on the silage diet.

Table 4: Impact of dietary treatment on milk yield and composition

	Treatment			
Item	GS	PC	SEM	*P*-value
Milk production (kg/d)				
Milk yield	28.02	27.33	0.724	0.510
Fat	1.27[a]	1.17[b]	0.031	0.032
Protein	0.96	0.94	0.019	0.341
Milk solids	2.24	2.11	0.046	0.055
Lactose	1.23	1.21	0.027	0.713
Casein	0.75	0.74	0.015	0.412
Milk quality %				
Fat	4.58	4.35	0.133	0.243
Protein	3.47	3.44	0.071	0.799
Lactose	4.47	4.49	0.014	0.351
Casein	2.79	2.76	0.055	0.726
Urea (% mg/l)	0.027[a]	0.024[b]	0.0007	0.008
SCC (cells/ml)[1]	27	29	3.613	0.064
ECM (kg) [2]	25.63[a]	22.64[b]	0.894	0.025

On week 6 of the experimental period, faecal samples were taken once a day for 5 consecutive days and urine samples were taken for 2 consecutive days to determine nitrogen and phosphorus balance. The results evaluating the impact of the press cake (PC) diet on N balance compared with grass silage (GS)

is shown in Table 5, and shows a significantly lower proportion of protein intake in the presscake diet (as expected due to some material being pressed out in the biorefinery). However, a significantly higher proportion of N in the PC diet ends in the milk, with a significantly lower amount of N being lost in excrement compared with the GS diet**. The increase in Nitrogen Use Efficiency is significant at 33% in the PC diet compared 27% in the GS diet**.

Table 5: The impact of press cake treatment on N balance

	Treatment			
Item	GS	PC	SEM	*P*-value
Intake (kg/d)				
Feed N	0.71[a]	0.60[b]	0.035	<0.0001
N output (kg/d)				
Milk	0.19	0.18	0.013	0.502
Faeces	0.21[a]	0.15[b]	0.018	<0.0001
Urine	0.29[a]	0.24[b]	0.014	<0.0001
N partitioning				
Milk	0.27[a]	0.33[b]	0.006	<0.001
Faeces	0.32[a]	0.27[b]	0.010	0.006
Urine	0.40	0.39	0.022	0.375
N excreted (%)	72.74[a]	66.96[b]	0.622	<0.0001
NUE (%)	27.25[a]	33.03[b]	0.622	<0.0001

The results of the impact of PC diet on P balance is shown in Table 6, and again shows a significantly lower level of P into in the PC diet compared with GS. Once again, a significantly higher proportion of the P ends up in the milk of PC diet with a **significantly lower amount of P being contained in excrement contained to GS. Overall, the results on potential to improve N and P balances are viewed very positively.**

Table 6: The impact of press cake treatment on P balance

	Treatment			
Item	GS	PC	SEM	*P*-value
Intake (kg/d)				
Feed P	0.095[a]	0.089[b]	0.0001	<0.0001
P output (kg/d)				
Milk	0.026	0.025	0.0007	0.286
Faeces	0.066[a]	0.046[b]	0.0035	<0.0001
Urine	0.0039	0.0034	0.00005	0.1836
P partitioning				
Faeces	0.68[a]	0.51[b]	0.023	<0.0001
Milk	0.26	0.25	0.0007	0.2864
Urine	0.030	0.027	0.0052	0.4255
P excreted (%)	72.63[a]	53.96[b]	2.57	<0.0001

Following the animal trial, an in vitro experiment was conducted to assess apparent digestibility, rumen fermentation, total gas and methane production, using the Rumen Simulation Technique (RUSITEC). In line with the in-vivo feed trials a 66% replacement rate of grass silage with presscake was used. The

results, presented in Table 7 below, show that the total volume of gas produced, and the total volume of methane is lower in PC diet, compared with the GS trial. The results showed an **approx. 15% reduction in methane emissions from the trial diet**, which should be further explored in future trials.

Table 7: In-vitro study of the effect of press cake treatment on apparent digestibility, rumen fermentation, total gas and methane production

	Treatments			
Item	GS[1]	PC[2]	SEM	*P*-value
DM disappearance % (s)	84.40	66.56	4.433	0.100
DM disappearance % (t)	77.85	65.76	3.346	0.158
Fermentation parameters				
pH	6.85	6.90	0.045	0.477
NH_3 (mmol/L)	1.68	1.45	0.371	0.674
Total gas production (l/d)	1.31	1.26	0.272	0.908
Methane (mmol/d)	6.61	5.71	1.248	0.638

Figure 10: Dairy Cattle Feeding Trial Pictures

Figure 11: RUSITEC Machine used to analyse rumen methane emissions

Overall, the results of presscake evaluation were extremely promising, demonstrating a sustainable, protein efficient, alternative to silage for cows. Further work could be undertaken to understand the full environmental impact of the trial findings and its implication to resolving emissions challenges in pasture-based agriculture. The results are further elaborated in Biorefinery Glas Deliverable 2.1. The results have been further published in the Journal of Livestock Science[5] (included as appendix), with one additional publication pending.

3.3.2 Protein concentrate pig feed trial data

The analysis of the grass protein concentrate co-product from the grass biorefinery as an alternative feeding source to baseline product soy bean in pig diets was led by the Barryroe Co-operative. Two trials on commercial pig farms were conducted during the project. One of the pig trials focused on wet feeding systems and the second focused on a dry feeding system. From the biorefinery demonstrations, a batch of the extracted grass protein was isolated for use within the first pig feed trial (wet feeding trial). The sample was analysed to understand its composition in advance of the feeding trial. The analysis, presented in Table 8 below, indicated a total solids concentration of 5.8% which was lower than anticipated. This may have been caused by some issues in the separation process during the demonstration phase, along with some additional washing water entering the system at the start of the biorefinery process. The total protein concentration was 2%, approximately 1/3 of overall solids. A more comprehensive analysis of both samples, including breakdown of amino acid composition is included in Biorefinery Glas Deliverable 2.4.

[5] E. Serra, M.B. Lynch, J. Gaffey, J.P.M. Sanders, S. Koopmans, M. Markiewicz-Keszycka, M.H. Bock, Z.C. McKay, K.M. Pierce, Biorefined press cake silage as feed source for dairy cows: effect on milk production and composition, rumen fermentation, nitrogen and phosphorus excretion and in vitro methane production, Livestock Science, Volume 267, 2023, 105135, ISSN 1871-1413, https://doi.org/10.1016/j.livsci.2022.105135.

Table 8: Composition of wet and dry protein feeds

Parameter	Wet Protein	Dry Protein
Crude Ash	0.5%	11.8%
Crude Fibre	0.1%	6.1%
Protein	2%	33.9%
Starch	0.3%	0%
Total solids	5.8%	87%

The first pig feed trial took place on a wet-feeding 2,000 sow unit in Co. Tipperary, which produces approximately 60,000 slaughter pigs annually. The farm is a fully integrated taking animals from birth to slaughter on the same site. The farm manufactures its own feed through an on-site feed mill. Due to the low dry matter composition of the initial grass protein sample, this was incorporated at just a 10% of the finished feed in the trial feed, replacing over 25% of barely in the diet and over 10% of the soya meal. The trial included 224 early finisher weaners and was conduct over a 14-day period. Pigs were weighed individually at the start, 7 days later, and at the end of the experiment (14 days). The pigs grew at an average of 900g/day with an average intake of 1.717 per head per day and a feed conversion of 1.91 for the trial. There is a significant change in the feed conversion from1.67 to 2.08 from Week 1 to Week 2, which not be usual. This is likely due to the dry matter inconsistency of the grass protein product which differed greatly from between storage containers. In hindsight, the containers should have been mixed together in one big tank and homogenised to reduce variation in DM coming from different farms. When observing the pens during the trial, a lot of clean troughs were visible throughout, indicating pigs were not fully fed to appetite. It is possible that dry matter content may have been overestimated which would negatively affect feed conversion efficiency also. Overall health was similar to other groups of pigs reared on conventional diets. The aroma of the liquid grass protein and the finished feed was pleasant, similar to grass silage. The feed was well received by the pigs, and animals ate well. The finished feed had a dark green colour and the faeces from the pigs consuming the product was a greenish colour. Dung consistency was normal. Pigs were clean and no difference noted compared to conventional diets.

In order to create dry protein, concentrate for our second feeding trial, different drying methods were trialled. Spray drying was first used and while the product initially separated well through centrifugation, the drying did not work as product lodged the walls of the spray dry becoming difficult to collect. A second batch of protein was then produced and dried using a Dorset dryer, which supports drying through a combination of heat and plate movement. This approach has previously been used in the drying of various wet biomass sources. This approach was successful in producing a high DM content green protein concentrate for use in our drying feeding trials. The analysis of this dry protein is also included in Table 8 above and shows a high DM content of almost 90% of which 34% is protein. This compares relatively favourably with various feed alternatives found in the literature and presented in Table 9 below.

Table 9: Crude fibre, crude protein, and amino acid profile of various feeding meals

Animal Feed Protein Sources	Crude Protein	Lysine	Methionine	Cysteine	Threonine	Crude Fibre
Soybean Meal	44 – 48	2.81 – 3.20	0.60 – 0.75	0.69 – 0.74	0.71 – 2.00	3.0 – 7.0
Sunflower Meal	24 – 44	1.18 – 1.49	0.74 – 0.79	0.55 – 0.59	1.21 – 1.48	12.0 – 32.0
Rapeseed Meal	34 – 36	2.00 – 2.12	0.67 – 0.75	0.54 – 0.91	1.53 – 2.21	10.0 – 15.0
Cottonseed Meal	24 – 41	1.05 – 1.71	0.41 – 0.72	0.64 – 0.70	1.32 – 1.36	25.0 – 30.0
Grass Protein Juice	2.0	0.1	0.03	0.01	0.07	0.1
Grass Protein Concentrate	33.9	1.81	0.65	0.18	1.5	6.1

The dry feeding pig trial ran over a 31-day period in January and February 2021 at a commercial pig farm in Barryroe Co-operative. This trial focused on late stage finishing weaner pigs entering second stage weaner accommodation aging 9 weeks old and weighing 20 kg on an average. The pigs were split into treatment groups and control groups with 54 pigs and 55 pigs respectively for approximately 30 days until slaughter. The control feed consisted of wheat, maize, barley, molasses, SBM, soy oil, soy hull and minerals in the recommended amounts. The treatment feed, on the other hand, comprised with green protein replacing a proportion of soybean meal, barley and wheat by 27.27%, 25% and 8% respectively and in comparison, to the control. The compositions of the treatment feed and control feed has been provided in Table 10. Weekly weigh-ins and feed intakes were recorded to allow the calculation of daily feed intake, average daily gain and feed conversion efficiency for each treatment.

Table 10: Composition of control and treatment feed

Raw Material	Control	Treatment
Barley	30.00	22.50
Maize	10.00	10.00
Wheat	25.00	23.00
Molasses	2.00	2.00
Hipro Soya	22.00	16.00
Grass Protein Pellets	-	15.00
Soya Hulls	1.00	1.50
Lactoflo	2.50	2.50
Soya Oil	3.70	3.70

100 Weaner+ Vita GP (3.8%)	3.80	3.80

During the dry feed trails, the daily feed intake was recorded at the end of every week. The treatment feed was very well received by the pigs, and they ate well. The average weight of the pigs at the start of the trials were recorded to be 1.079 kg for the control diet and 1.132kg for the treatment diet. There was a steady increase in the feed intake for both control and treatment diets as expected. During the first week, the feed intake for the control feed was recorded to be 0.991 kg/d. In comparison, this figure was 1.022 kg/d for the treatment diet. As the trial progressed the difference between the daily feed intake for the control diet and the treatment diet increased considerably. By the end of the trial, the daily feed intake for the treatment diet (1.512 kg/d) was 8% higher than the control diet (1.400 kg/d).

For the dry feed trials, the weaner pigs were weighed individually at the start of the trial and at the end of the week thereafter. The superiority of the treatment diet over the control can be observed from ADG comparisons. On the control diet, the pigs gained 0.592 kg/day during the first week. This rate increased as the trial progressed with an average daily gain of 0.646 kg/day after the second week, 0.699 kg/day after the third week and 0.682 kg/day at the end of the trial. On the other hand, the average weight gain of pigs on the treatment diet started slowly at 0.577 kg/day by the end of the first week but increased substantially as the trials progressed. The average weight gain by the end of the second week was reported to be 0.683 kg/day, which increased to 0.729 kg/day. By the end of the trial a high average weight gain of 0.742 kg/day was achieved. This figure was 6.44% higher than the final weight gain achieved in control sample. An overview of the main parameters daily feed intake, feed conversion efficiency and average daily gain is included in Table 11 below.

Table 11: Daily Feed Intake, Feed Conversion Efficiency and Average Daily Gain of weaners on treatment and control diet

Date of Weighing	Daily Feed Intake (kg/d)		Feed Conversion Efficiency		Average Daily Gain (kg/day)	
	Treatment	Control	Treatment	Control	Treatment	Control
Period 1	1.022	0.991	1.77	1.67	0.577	0.592
Period 2	1.247	1.182	1.83	1.83	0.683	0.646
Period 3	1.386	1.301	1.90	1.86	0.729	0.699
Period 4	1.512	1.400	2.04	2.05	0.742	0.682

Figure 12: Pig feeding trials pictures (i) storage and treatment of protein concentrate (ii) pig feed trial

Overall the performance of our grass biorefinery co-product has been very positive, showing that through a more efficient use of grass, both cows and pigs could be fed. In particular, the use of dry grass protein concentrate shows a lot of potential to replace existing pig feed ingredients such as soyabean as well as barley. The results are elaborated in Biorefinery Glas Deliverable 2.4. These results have also been publishing in Clean Technologies Journal[6] which is included in appendix. Future work could example how other monogastric animals, such as chickens, or even humans could utilise this protein. Additionally, the environmental impact of replacing soyabean imports with grass protein could be explored.

3.3.3 Data from pre-commercial validation of fructo-oligosaccharides from grass whey

The analysis of fructoligosaccharides (FOS) from grass whey was conducted by MTU. Apprxoimately 60 litres of grass whey was collected from the biorefinery trials. This represents the by-product of grass following extraction of press-cake (cattle feed) and protein concentrate (pig feed). At MTU, the whey was refrigerated at 4°C, then concentrated in a rotavapor at 50°C at 75 rpm to approximately 1/3 of the volume. The concentrated samples were then filtered using a Whatmann (10 µm) filter paper to remove all solid particles. After filtration the samples were centrifuged at 10000 rpm for 20 minutes, with supernatants collected for further analysis. An Amicon stirred cell (Figure 13) was used to separate the concentrated grass whey into fractions containing sugars of smaller degrees of polymerization. The two filters used for analysis were 10000 MWCO and 1kDa cellulose filters, no separation occurred while using 10000 MWCO filter. 1 kDa filters simulating nano-filtration process in large scale was used to separate the whey into FOS enriched samples.

The validation of HPLC-RI method for the quantification of the main sugars' glucose; and FOS (1-kestose, nystose, and 1F-fructofuranosylnystose) were performed. The concentrations of various oligosaccharides is shown in Table 12; the total concentration of sc-FOS is around 4.65 g/L and glucose

[6] Ravindran, R., Koopmans, S., Sanders, J.P., McMahon, H. and Gaffey, J., 2021. Production of green biorefinery protein concentrate derived from perennial ryegrass as an alternative feed for pigs. Clean Technologies, 3(3), pp.656-669.

concentration is 4.79 g/L, respectively. From earlier analysis performed by project partner GRASSA, it was shown that depending on the weather conditions and time of mowing, the concentrations of FOS in grass whey varied between 4 to 8 g/L, this value is in line with the total concentration of sc-FOS recorded in this study (4.65 g/L).

Table 12: Concentration of Glucose and sc-FOS calculated from HPLC-RI

Saccharide	Concentration (mg/ml)
Glucose	4.79
Nystose	2.53
Fructofuranylnystose	1.14
2-Kestose	0.98

The prebiotic potential of the extracts was measured via growth tests of individual probiotic strains of 3 Lactobacilli and 2 Bifidobacteria's, respectively, using Grass FOS as the sole carbon source. The control/baseline prebiotics analysed were 90% pure FOS and inulin. The results in Table 13 below of the prebiotic analysis show that grass FOS had a beneficial effect on probiotic strains by supporting their growth in medium. It indicated a positive prebiotic index (PI) with all probiotics tested, and the overall prebiotic indices were very similar to that of commercial FOS standards. The organic acid analysis of the prebiotic media enriched with grass FOS were performed and it substantiated the results of growth tests. Overall, Grass FOS had a beneficial effect on probiotic strains by supporting their growth in medium. It indicated a positive prebiotic index (PI) with all probiotics tested. The fermentation of Grass FOS was manifested by the production of organic acids (mainly lactic acid and acetic acid) and in turn led to a decrease in pH of growth medium.

Table 13: Prebiotic indices of various bacterial strains on various sugar sources

Strain/Prebiotic	Grass FOS	Commercial FOS	Inulin
Lactobacillus acidophilus	1.12±1.3	1.09±0.8	0.88±0.6 *
Lactobacillus fermentum	1.67±0.9	1.32±0.8	3.67±0.7 *
Lactobacillus plantarum	1.44±0.5	0.78±0.7 *	-0.354±0.6 *
Bifidobacterium animalis	1.89±0.45	2.05±0.11	5.98±0.41*
Bifidobacterium breve	3.65±0.98	4.01±1.12	2.76±0.23 *

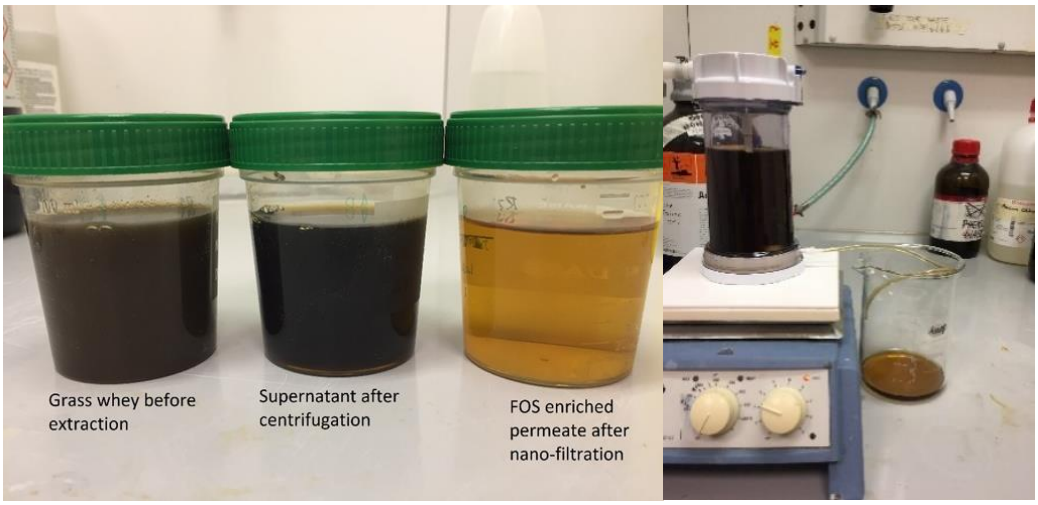

Figure 13: Photos from FOS extraction process (i) grass extracts after various separation processes (ii) stirred cell apparatus used in grass separation

Overall, the results of the analysis of FOS contained in grass are very positive, with grass-based FOS displaying a comparable prebiotic potential to market FOS sources such as inulin from chicory. This represents a potentially major new use of grass in the development of animal nutrition or human nutraceutical products. It also adds a lot of value add to the approach. More details on this work are available in Biorefinery Glas Deliverable 2.5. The results from this analysis an currently pending peer-review publication.

3.3.4 Data analysis of bio-fertiliser potential of residual grass whey

The analysis of the bio-fertiliser potential from grass whey, a residue from the biorefinery process was conducted by Carbery farmers on their land. The product was spread on bare ground straight after cutting grass (which was used in the biorefinery demonstrations) and monitored over a six-week period. Three farmers monitored the response of grass growth to the grass whey and recorded their results. To do this, the farmers split paddocks into 3 strips. The first received grass whey, the second received slurry or soiled water and the third was a control plot. All nutrients were spread with a trailing shoe. The farmers took grass measurements over the six weeks and were able to compare the responses to the various nutrient sources spread. Tables 14, 15 and 16 below outline the growth response:

Farm A:

Table 14: Bio-fertiliser results from Farm A

Whey	Slurry (undiluted)	Control
1156 KG/Dm/Ha	1292 Kg/DM/Ha	782 Kg/DM/Ha
Spread 30 M^3/Ha	Spread 30 M^3/Ha	Spread 30 M^3/Ha
Samples taken 6 weeks after spreading	Samples taken 6 weeks after spreading	Samples taken 6 weeks after spreading

Points from farm A: The ground was slow to recover initially which explains the comparably lower growth rates across all plots. The reason for this was that the grass was quite strong due to the delayed

time of cutting making it quite stemmy. However, there is a noticeable difference in growth between the whey and control strips and little difference between the whey and slurry strips which suggests that there are nutrients available and the bio-fertiliser whey stimulates grass growth.

Farm B:

Table 15: Bio-fertiliser results from Farm B

Whey	Slurry (Diluted)	Control
1806 KG/Dm/Ha	1849 Kg/DM/Ha	1333 Kg/DM/Ha
Spread 30 M^3/Ha	Spread 30 M^3/Ha	Spread 30 M^3/Ha
Samples taken weekly for 6 weeks	Samples taken weekly for 6 weeks	Samples taken weekly for 6 weeks

Points from farm B: Like farm A, the grass was initially quite stemmy but not as strong. Growth response to both the whey and slurry was similar and greater than the control plot. Similar to farm A, this suggests considerable nutrients are available for uptake by the plants in the grass whey. The farmer noted a lush green colour in comparison to the slurry plot from grass whey.

Farm C:

Table 16: Bio-fertiliser results from Farm C

Whey	Slurry (Diluted)	Control
1700 KG/Dm/Ha	1710 Kg/DM/Ha	1120 Kg/DM/Ha
Spread 30 M^3/Ha	Spread 30 M^3/Ha	Spread 30 M^3/Ha
Samples taken weekly for 4 weeks	Samples taken weekly for 4 weeks	Samples taken weekly for 4 weeks

Points from farm C: Similarly, to farm B, growth responses were excellent to the grass whey and there was little difference with the slurry plot. Both exceeded the control plot. The farmer noted that it was pleasant to spread the grass whey due to the sweet smell in comparison to spreading animal slurry.

The phosphorous (P) and potassium (K) values of grass whey are 7433.69 mg/kg and 87057 mg/kg respectively. High concentrations of K and low concentrations of P make it quite suitable for using it as fertiliser (similar to slurry or diluted slurry), however from analysis it does contain easily biodegradable matter which has potential to cause soil acidification. Given that Irish soils are already acidic, it would be necessary to spread the whey immediately after production otherwise whey may need to be stabilised through anaerobic digestion.

Overall, the grass whey has sufficient nutrient value to act as a bio fertiliser for Irish pasture systems. This could be utilised in systems where integration of biorefinery with anaerobic digestion is not under consideration. The results are further elaborated in Biorefinery Glas Deliverable 2.6.

3.3.5 Data analysis of biogas/biomethane potential of residual grass whey

Analysis of the biogas/biomethane potential of residual grass whey took take place in two phases;

- an initial analysis of biogas/biomethane potential of grass whey, referred to as whey, prior to extraction of fructo-oligosaccharides (i.e. following extraction of press-cake and protein)
- a second analysis of biogas/biomethane potential of grass whey, post FOS extraction (see section 3.3.3), referred to as de-FOS whey (i.e. following extraction of press-cake, protein and FOS)

Using this approach, allows a better assessment regarding the potential of anaerobic digestion to implemented at various process points.

- An additional analysis was also included to assess the biogas and biomethane potential of presscake

Based on the analysis, conducted in collaboration with Celignis Analytical, biomethane potential of grass whey was somewhat lower when compared with silage (36 L/Kg for de-FOS whey, compared to approx. 78.8 L/Kg for grass silage) – see table 17 below. This was expected, given that 2-3 products (ruminant feed, monogastric feed and FOS) have already been extracted from the grass prior to digestion. Despite this, our analysis indicates that grass whey is a very suitable feedstock for anaerobic digestion, with significant biogas/biomethane potential (up to 85% biomethane in the case of de-FOS whey). Based on the analysis, whey had a very short residence time, converting to biogas/biomethane in a few days or less, and would likely cause few processing challenges. By comparison grass silage usually requires a residence time of 21-30 days, often requiring pre-treatment to break down feedstock prior to digestion. This approach of using whey for anaerobic digestion is likely to be only suitable for centralized, medium scale green biorefineries, rather than mobile systems. The biogas and biomethane potential results of de-FOS grass whey combined with the positive results of FOS extracted and analysed during the project (see section 3.3.3) indicates that a coupling of these processes within a green biorefinery system offers much potential. Given that there are some heating requirements within the green biorefinery system (e.g. in protein separation and drying), utilising the whey or de-FOS whey waste stream to create heat and electrical energy in order to supply the process energy, will help the process to become more self-sufficient from an energy point of view, and will also reduce or eliminate the carbon footprint of the process.

We also investigated the use of presscake as a feedstock for anaerobic digestion. The results, in table 18 below, indicate that presscake could be a suitable feedstock for biogas or biomethane production, within a process which allows the co-production of protein and other ingredients. Press cake has a fresh weight biomethane potential of 113L/Kg fresh weight, which is ahead of silage. Given the growing interest in biogas as a diversification opportunity for Irish farmers, we have shown, through Biorefinery Glas that the integration of biorefinery and biogas in order to satisfy both feed, ingredient and energy markets may offer a pathway which provides both economical and environmental benefits.

Table 17: Summary of biogas and biomethane potential of grass biorefinery de-FOS whey

	Biogas potential (l/Kg VS)	BMP (l/Kg VS)	Biogas potential (l/Kg DM)	BMP (l/Kg DM)	Biogas potential (l/Kg Fresh Weight)	BMP (l/Kg Fresh Weight)
AV.	597.37	520.28	478.23	416.51	41.46	36.11
Rep 1	600.33	522.71	480.60	418.46	41.66	36.28

Rep 2	616.86	536.64	493.83	429.61	42.81	37.24
Rep 3	574.92	501.49	460.25	401.47	39.90	34.80
SD	21.13	17.70	16.92	14.17	1.47	1.23

Table 18: Summary of biogas and biomethane potential of grass biorefinery presscake

	Biogas potential (l/Kg VS)	BMP (l/Kg VS)	Biogas potential (l/Kg DM)	BMP (l/Kg DM)	Biogas potential (l/Kg Fresh Weight)	BMP (l/Kg Fresh Weight)
AV.	517.07	303.95	492.94	289.77	192.25	113.01
Rep 1	482.41	283.62	459.89	270.38	179.36	105.45
Rep 2	552.50	324.70	526.72	309.54	205.42	120.72
Rep 3	516.31	303.55	492.21	289.38	191.96	112.86
SD	35.05	20.54	33.42	19.58	13.03	7.64

The results indicate strong potential to integrate biogas and biorefinery systems to create multiple value-added products using a process which requires minimal fossil-based energy. There are multiple streams which could be used for anaerobic digestion. The results are further elaborated in Deliverable 2.6. These results have been published in peer-review in the Journal of Clean Technologies[7] (included as appendix). Further work could explore the scale up of integrating the anaerobic digestion processing of grass whey, while evaluating the full potential environmental benefits of such an integrated system.

3.4 Key Performance Indicators

A summary of Biorefinery Glas expected return and target KPI's versus the achieved performance is provided in Table 19 below.

Table 19. Target and Achieved KPIs of Biorefinery Glas

KPI#	Expected return	Target KPI	Actual performance
KPI1	Demonstration of Small-scale Grass Biorefinery with farmers in the South West	Minimum of 4 farms participating directly in demonstrations/trials. 2 demonstrations per farm (1 in Summer, 1 in late Autumn). In	The project demonstrated on 5 participating dairy farms, and included 2 additional pig farmers as part of the analysis.

[7] Ravindran, R., Donkor, K., Gottumukkala, L., Menon, A., Guneratnam, A.J., McMahon, H., Koopmans, S., Sanders, J.P. and Gaffey, J., 2022. Biogas, biomethane and digestate potential of by-products from green biorefinery systems. Clean Technologies, 4(1), pp.35-50.

		total 8 demos – producing products for feed trials, field trials and validation.	
KPI2	Producing samples of fodder and value-added products for trials	112 tons fresh grass input (in each of two trials – 1 summer, 1 late Autumn), producing 33.6 tons press cake silage, 4.25 tonnes protein concentrate, 70 tonnes of whey containing 1.9 tonnes of carbohydrates (30-70% fructo-oligosaccharides) and nutrients	Based our processed material from the demonstration activities, we estimate that the actual processed material was largely in line with projected.
KPI3	Product Validation Trials	4 products analysed	5 products analysed (i) press cake replacement for silage, (ii) grass protein concentrate replacing soyabean meal (iii) FOS replacing on-the-market prebiotics (iv) grass whey as fertiliser (v) grass whey as biogas substrate
KPI4	Increase in protein efficiency	Increase in usable protein per ha by 40%	Given that cows fed with press cake largely upheld their milk productivity, while pigs few with the separated protein gain comparable weight to control pigs, we can assume that this KPI has been achieved.
KPI5	Improving sustainability of agriculture	Reduction in N and P of cattle excrement by 25% each, reducing imports of soybean feed	While the reduction in phosphorous losses was in line with the projected 25% for cows on a press cake diet, the reduction in nitrogen losses were lower at approximately 10% compared with cows on a silage diet.
KPI6	Knowledge Exchange Activities	8 on-farm demonstrations including training of farmers. Min 3 Workshop and Demonstration activities with Carbery farmers, on operational and sustainability aspects, business case and financial aspects, product validation results. 2 onboarding activities. 1 National Demo day for farmers Nationwide. National conference with sharing of results. At least 10 external events participating.	This achieved KPI is in line with expect KPI. A summary of events is provided later in this report.
KPI7	Number of unique visits to the project web portal (by the end of the project)	> 5000	>5000
KPI8	Number of followers in social media (Facebook, LinkedIn, YouTube and Twitter)	> 600	1,424

KPI9	Biorefinery Glas YouTube channel	8 videos/ > 1000 views	11 videos/9344 views
KPI10	Biorefinery Glas Promotional Material distributed during project/ external events	> 500	Approx. 300. We distributed approximately 300 promotional packs at our open day, but due to COVID occurring during year 2, many of our dissemination activities took place online, making further leaflet distribution challenging.
KPI11	EIP-AGRI practice abstracts (no of abstracts/ copies distributed through dissemination channels)	1 / 1000	1 available through our website. Physical distribution was hampered by COVID 19.
KPI12	Audio-visual material (short videos, demonstration audio-visual showcases, farmer stories)	8 videos	11 videos.
KPI13	Number of Newsletters	8	8 Newsletters.
KPI14	Number of press releases	5	13
KPI15	Number of fact-sheets	5	5 available through our website.
KPI16	Number of external national demonstration (field visit) events	1	1
KPI17	Number of participants in the demonstration event	> 80	300
KPI18	Number of external events participated in	> 10	24
KPI19	Final Dissemination conference participants	> 50	140 registered attendees (Zoom webinar due to COVID)
KPI20	Number of synergies with other EIP-AGRI and EU projects	> 5	7

3.5 Closing Evaluation

The Biorefinery Glas project has made a significant contribution to boosting awareness of the bioeconomy among farmers in Ireland, along with other stakeholders. It has been a first demonstration of grass biorefining, and has show the potential that can be achieved from Ireland's vast grasslands, by converting this to feed for cows along with additional co-products which can have economic and environmental benefits. All of the tested products have performed well against market products, indicating the large potential to develop this opportunity further. Implementing the technology on Irish farms has greatly improved the visibility of the technology among farmers and the wider public, and has helped to improve the viability of this opportunity to be further developed within the farming community.

3.6 Value For Money

In value for money terms, the project has enabled the testing of an existing and adapted mobile biorefinery in South West Ireland across multiple farms. In addition to testing of technology, the project also included a comprehensive analysis of the different co-products, and the completion of a business case, as well as financing and policy analyses. Finally, the project included a comprehensive dissemination and engagement package. The use and adaption of existing technology from the

Netherlands to Irish farms, has reduced the costs involved in building new technology from scratch. This has enabled a more detailed validation of the products and opportunities, including comprehensive animal feeding trials along with lab-based analysis. This detailed analysis has provided a sound platform for assessing the suitability of the feedstock for Irish agriculture. The use of technology on multiple farms has enabled greater visibility for the biorefinery. The project has over-delivered in terms of products assessed, and has strongly delivered both in scientific validation (verified by its peer-reviewed publications in appendix), and the level of public awareness of the project (e.g., through channels such as Big Week On the Farm and Farmers Journal).

4.0 FINANCIAL REPORT

The total spend from the Biorefinery Glas project was €805,712.57 which was below the projected project budget from the proposal phase of €940,498. The spent across different categories is outlined in Table 20. All categories of costs were lower than anticipated. Personnel costs accounted for the larger share of costs which was anticipated. Other direct costs included the biorefinery demonstration activities along with the various consumables associated with the completion of trial work. Travel was lower than anticipated since the project in-person activities were impacted by the COVID 19 Pandemic. An overview of the spend from each partner across categories is outlined in Tables 21-25 below.

Table 20. Total Project spend across different cost categories

Sub-Heading	**Total Paid**
Personnel Costs	€367,671.06
Travel	€13,933.52
Other Direct Costs	€172,348.35
Overheads	€123,032.70
Subcontracting & Advisory Board	€128,726.94
Totals	€805,712.57

Table 21. Total MTU spend across different cost categories

Sub-Heading	Total Paid	**Total VAT Paid**
Personnel Costs	€185,454.04	
Travel	€4,275.22	
Other Direct Costs	€11,133.33	€1,422.82
Overheads	€50,215.66	
Subcontracting & Advisory Board	€38,856.77	€6,927.43
Totals	€289,935.02	**€8,350.25**

Table 22. Total Carbery spend across different cost categories

Sub-Heading	Total Paid	**Total VAT Paid**
Personnel Costs	€66,255.00	
Travel	€1,736.01	
Other Direct Costs	€42,322.27	€31.09
Overheads	€16,546.99	€0.00
Subcontracting & Advisory Board	€6,242.50	€742.50
Totals	€133,102.77	**€773.59**

Table 23. Total GRASSA spend across different cost categories

Sub-Heading	Total Paid	**Total VAT Paid**
Personnel Costs	€39,390.05	€232.05
Travel	€5,079.56	
Other Direct Costs	€76,292.28	€4,840.46
Overheads	€24,105.96	€0.00
Subcontracting & Advisory Board	€70,827.67	€12,327.21
Totals	€215,695.52	**€17,399.72**

Table 24. Total UCD spend across different cost categories

Sub-Heading	Total Paid	**Total VAT Paid**
Personnel Costs	€61,121.97	
Travel	€1,922.97	
Other Direct Costs	€29,367.21	€1,504.17
Overheads	€27,723.65	
Subcontracting & Advisory Board	€0.00	
Totals	€120,135.80	**€1,504.17**

Table 25. Total Barryroe spend across different cost categories

Sub-Heading	Total Paid	**Total VAT Paid**
Personnel Costs	€15,450.00	€0.00
Travel	€919.76	
Other Direct Costs	€13,233.27	€1,848.93
Overheads	€4,440.45	€0.00
Subcontracting & Advisory Board	€12,800.00	€0.00
Totals	€46,843.48	**€1,848.93**

5.0 LESSONS LEARNED

Based on the Biorefinery Glas results, it is understood that we can produce a number of different products from grass. Grass cannot only be used for cattle feed, but also used to co-produce pig feed, pre-biotics, bioenergy and fertiliser. These technologies and products have now been tested in an Irish environment in collaboration with primary producer stakeholders. This provides a foundation for the further development of grass biorefinery in Ireland. Aside from understanding the potential of this new value chain for rural Ireland and the livestock sector, a number of additional learnings came from the project. The involvement of farmers in this multi-actor bioeconomy project has been enormously impactful, as it allowed the other team members to understand the logistical challenges and limitations to implementing a mobile grass biorefinery on Irish farms. The team members benefited from the farmer know-how concerning the logistical aspects of harvesting and mobilising grass for biorefining in a model which also requires the maintenance of existing farm enterprises (e.g., provision of feed for cattle). In addition to identify the practical challenges of implementing the biorefinery approach, the collaboration of farmers within the demonstration has been hugely beneficial also in order to improve the visibility of the technology among the farming community. The collaboration with Carbery, Barryroe and the associated farmers involved, help to increase the interest among their peers, through the farmer networks. Carbery also helped in identifying key channels for targeting farmers in order to achieve maximum impacts (e.g., the use of farm walks within our demonstration day activities). At the same time, researchers brought scientific knowledge to optimise the process under demonstration and analyse the products produced in a way that supports farmers towards the subsequent larger uptake/deployment of technology. When dealing with the harvesting of grass for biorefinery purposes, care should be taken to set timelines (e.g., project state date) at a time which takes into account months of the year when grass can best be harvested. In the case of the Biorefinery Glas project, which stated in March 2019, this made it quite challenges to put all the necessary plans in place to process during months when grass growth was strongest, and some of our demonstration activities fell into August. Care must all be taken to ensure the alignment of production with the validation trials to be conducted, and to ensure that co-products do not deteriorate in quality. Adequate treatments should be applied to help with preservation when appropriate.

6.0 ACTIONS TO CARRY FORWARD

The work of Biorefinery Glas has been already built upon through the SFI Farm Zero C project which has undertaken further trials of the different green biorefinery products, including the higher displacement of 50% of soya bean with grass protein in pig diets[8]. Recently DAFM have award the investment of €3 million to UCD and MTU to further advance this work in collaboration with Carbery[9]. This development, which will be based at Shinagh Farm in Cork, will help to scale the activities of Biorefinery Glas in a farm setting, while also advancing the work to broaden its feedstock scope, and to target new higher value materials which can be produced from grass. It will also improve the integration of the biorefinery and AD system demonstrated through the Biorefinery Glas model. The collaboration will continue to promote the multi-actor approach demonstrated through Biorefinery Glas. Within Farm Zero C we will further analyse how the biorefinery and AD model can help farms to contribute to the governments target of net zero emissions, and specifically contribute to the targets set out for the agriculture sector, by using the farm grass more efficiently to produce products which can offset products such as protein or energy. In addition, the learning of Biorefinery Glas, have help to inspire many new attempts to develop grass biorefinery and small-scale biorefineries in a farm setting. New projects such as MainstreamBIO and RuralBioUp are currently working with farmers in Ireland to promote and support the adoption of small-scale bio-based solutions among primary producers, and this is something in which Biorefinery Glas partners are actively engaged.

[8] Gaffey, J., O'Donovan, C., Murphy, D., O'Connor, T., Walsh, D., Vergara, L.A., Donkor, K., Gottumukkala, L., Koopmans, S., Buckley, E. and O'Connor, K., 2023. Synergetic Benefits for a Pig Farm and Local Bioeconomy Development from Extended Green Biorefinery Value Chains. Sustainability, 15(11), p.8692.

[9] https://www.irishexaminer.com/farming/arid-41071235.html

7.0 DISSEMINATION OF PROJECT FINDINGS

During the course of the project Biorefinery Glas has been disseminated widely both nationally and internationally, through conferences, news media and social media.

7.1 Conferences, Workshops and Speaking Engagements

Members of the Biorefinery Glas OG have presented the project to approximately 3000 stakeholders at a variety of conferences, workshops and meetings, both nationally and internationally. These include:

International

- EIP-Agri Workshop: Opportunities for farm diversification in the circular bioeconomy, Vilnius Lithuania[10]
- Agri-Innovation Summit, 2019, Normandy, France[11]
- EIP-Agri Workshop: Small is smart: Innovative Solutions for small agricultural and forestry holdings, Bucharest, Romania[12]
- Hightech meets Biomass, Venlo Netherlands[13]
- 2nd Future Food Forum, Zhangzhou, Fuijan, China[14]
- Industrial Biotechnology Innovation Conference, 2020, Glasgow Scotland[15]
- International Working Group Meeting on Alternative Pastureland Use, New Zealand (via Skype)
- Green Biorefinery: A Green Deal for Agriculture, Presentation to EU Parliament, Online[16]
- The Future of Food: Unlocking the benefits of Scotland's Circular Bioeconomy, Zero Waste Scotland, Online[17]
- European Federation for Animal Science (EAAP) 2021 Conference, Online[18]

National

- Teagasc Seminar: Alternative pastureland-use strategy[19]
- Biogas Opportunities for South West Region
- AgroCycle – A Circular Economy for the Agri-Food Sector
- Enabling Project Meeting
- BBI Info Day 2019
- National Ploughing Championships 2019[20]
- Open Policy Debate on Future Agri-Food Strategy

[10] https://ec.europa.eu/eip/agriculture/en/event/eip-agri-workshop-opportunities-farm
[11] https://ec.europa.eu/eip/agriculture/en/event/agri-innovation-summit-2019
[12] https://ec.europa.eu/eip/agriculture/en/event/eip-agri-workshop-small-smart-innovative-solutions
[13] https://mailchi.mp/2cd6abcefa04/hightech-meets-biomass-10-10-2019
[14] https://www.agroberichtenbuitenland.nl/actueel/nieuws/2019/12/17/2nd-future-food-forum-zhangzhou-development-zone-fujian
[15] http://www.ibioic.com/news_and_events/annual_conference/conference_registration_and_programme/d1145/
[16] https://cbio.au.dk/arrangementer/webinars-on-solutions-for-green-transition/green-deal/presentations-and-streaming/
[17] https://www.zerowastescotland.org.uk/content/future-food-unlocking-benefits-scotlands-circular-bioeconomy
[18] https://meetings.eaap.org/
[19] https://www.teagasc.ie/news--events/news/2019/grass-as-a-biomass-source.php
[20] https://biorefineryglas.eu/biorefinery-glas-at-the-2019-national-ploughing-championships/

- The Sustainability Challenges facing the Irish Dairy Sector (ICOS)[21]
- Shannon ABC Research Colloquium, Institute of Technology, Tralee
- Shannon ABC Research Colloquium, Limerick Institute of Technology
- Resource Recovery from Dairy Industry Effluent[22]
- BBI Info Day 2020
- GoGrass - Exploiting grassland potential in the EU Circular economy - as part of Irish Bioeconomy Week 2020[23]
- Value of the Irish Bioeconomy, Irish Rural Link Webinar Series[24]

Figure 14: Some of Biorefinery Glas national and international speaking engagements

7.2 Press releases and media coverage

The Biorefinery Glas project has featured widely in news media including television, online and printed media. In particular, the project has featured in media channels which are specifically targeted towards farmers, a key dissemination audience for the project. A list of some of the media outlets featuring the project include:

- RTE's Big Week on the Farm (TV)[25]
- Irish Farmers Journal Article 1[26]
- Irish Farmers Journal Article 2[27]

[21] https://www.ucc.ie/en/fitu/courses/event-cpdtrainingcourse/#programme-of-events
[22] https://newtrients.ucc.ie/wp-content/uploads/sites/69/2019/11/Newtrients-Workshop-Flyer.pdf
[23] https://irishbioeconomy.ucd.ie/event/go-for-grass-exploiting-grassland-potential-in-the-eu-circular-economy/
[24] https://www.irishrurallink.ie/in-conversation-with-irish-rural-link-webinar-series-rural-innovation/
[25] https://biorefineryglas.eu/biorefinery-glas-on-big-week-on-the-farm/
[26] https://www.farmersjournal.ie/cork-project-turning-grass-juice-into-fertiliser-and-protein-478748
[27] https://www.farmersjournal.ie/watch-squeezing-value-out-of-grass-475602

- Irish Farmers Journal Article 3[28]
- AgriLand[29,30]
- The Irish Examiner[31]
- The Southern Star[32]
- Irish Farmers Monthly Magazine
- The Kerryman
- Feed Navigator Article 1[33]
- KPMG Agri Business Report[34]
- Feed Navigator Article 2[35]
- Old Moore's Almanac[36]

Figure 15: Some of media channels which have featured the Biorefinery Glas project

[28] https://www.farmersjournal.ie/plant-proteins-could-be-a-big-focus-in-farm-to-fork-strategy-532424
[29] https://www.agriland.ie/farming-news/biorefinery-the-potential-for-farmers-to-diversify-their-business-in-the-future/
[30] https://www.agriland.ie/farming-news/processing-grass-to-extract-valuable-constituents-in-cork/
[31] https://www.irishexaminer.com/breakingnews/farming/how-farms-could-get-40-more-usable-protein-from-grass-933569.html
[32] https://www.southernstar.ie/news/biomass-refinery-will-go-on-trial-in-west-cork-next-may-4165874
[33] https://www.feednavigator.com/Article/2019/05/16/Project-looks-to-extract-high-value-products-from-grass
[34] https://home.kpmg/ie/en/home/insights/2020/05/agribusiness-report-2020-ifj-kpmg.html
[35] https://www.feednavigator.com/Article/2021/03/04/Feed-protein-from-grass-Do-green-biorefineries-have-a-future-in-Ireland
[36] https://oldmooresalmanac.com/biorefinery-glas-a-brighter-future-for-irish-farmers/

7.3 Social Media

The official Biorefinery Glas website (www.biorefineryglas.eu) was launched in May 2019. The website contains a range of information relating to the Project including: a general introduction to the project; partners and contact details; a section on news and events, a project documents section which updates as documents are developed; a video section containing digital stories developed by each of the farmers from the demonstrations on their farm; fact sheets; a gallery section which provides a diary update of the project using photos. Where public, the project deliverables are available for download through the website. Since its launch the Biorefinery Glas website has been regularly updated and promoted through project social media channels and external channels.

Biorefinery Glas also have an active presence on social media, with Facebook (@BiorefineryGlas), Twitter (@BiorefineryGlas), LinkedIn (@Biorefinery Glas) and Youtube (Biorefinery Glas) pages. These social media accounts have been regularly updated with project events and news features and are the main source of updating interested parties on on-going project developments. Relevant posts from third parties, in Ireland and overseas, working in the area of bioeconomy, particularly as it relates to the agriculture sector have also been posted to our social media pages.

The Biorefinery Glas Twitter page currently has nearly 800 followers, while on LinkedIn the project has over 600 followers and over 100 followers on facebook.

The Biorefinery Glas Youtube profile currently contains 12 videos with 71 subscribers. The page features official project videos and videos from the Biorefinery Glas Digital Storytelling Initiative. The Biorefinery Glas Digital Storytelling Initiative has been a key aspect of our dissemination among farmers, with farmers who participated in demonstrations (dairy biorefinery trials and pig feeding trials) documenting their experiencing of the initiative with a personal video. To date the videos have received over 9000 views with some videos having over 2000 individual videos.

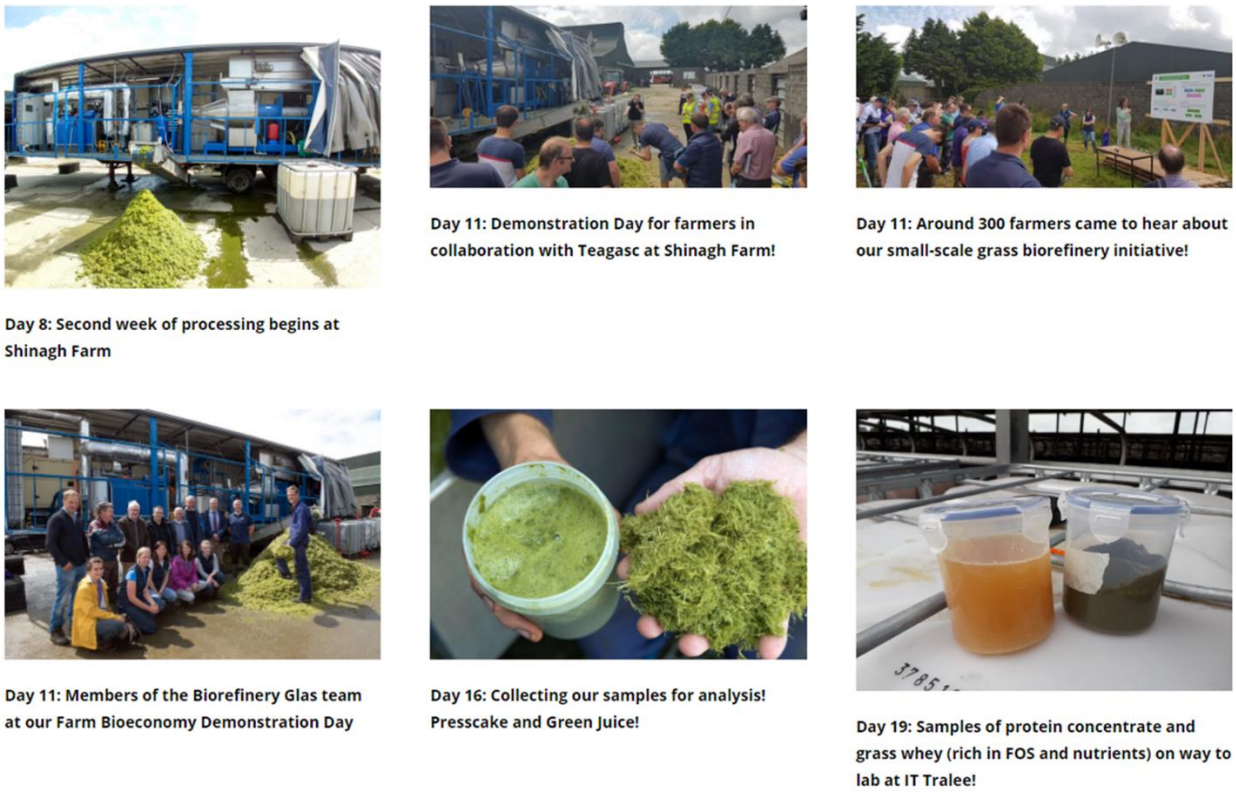

Figure 16: Biorefinery Glas Photo Gallery[37]

[37] https://biorefineryglas.eu/gallery/

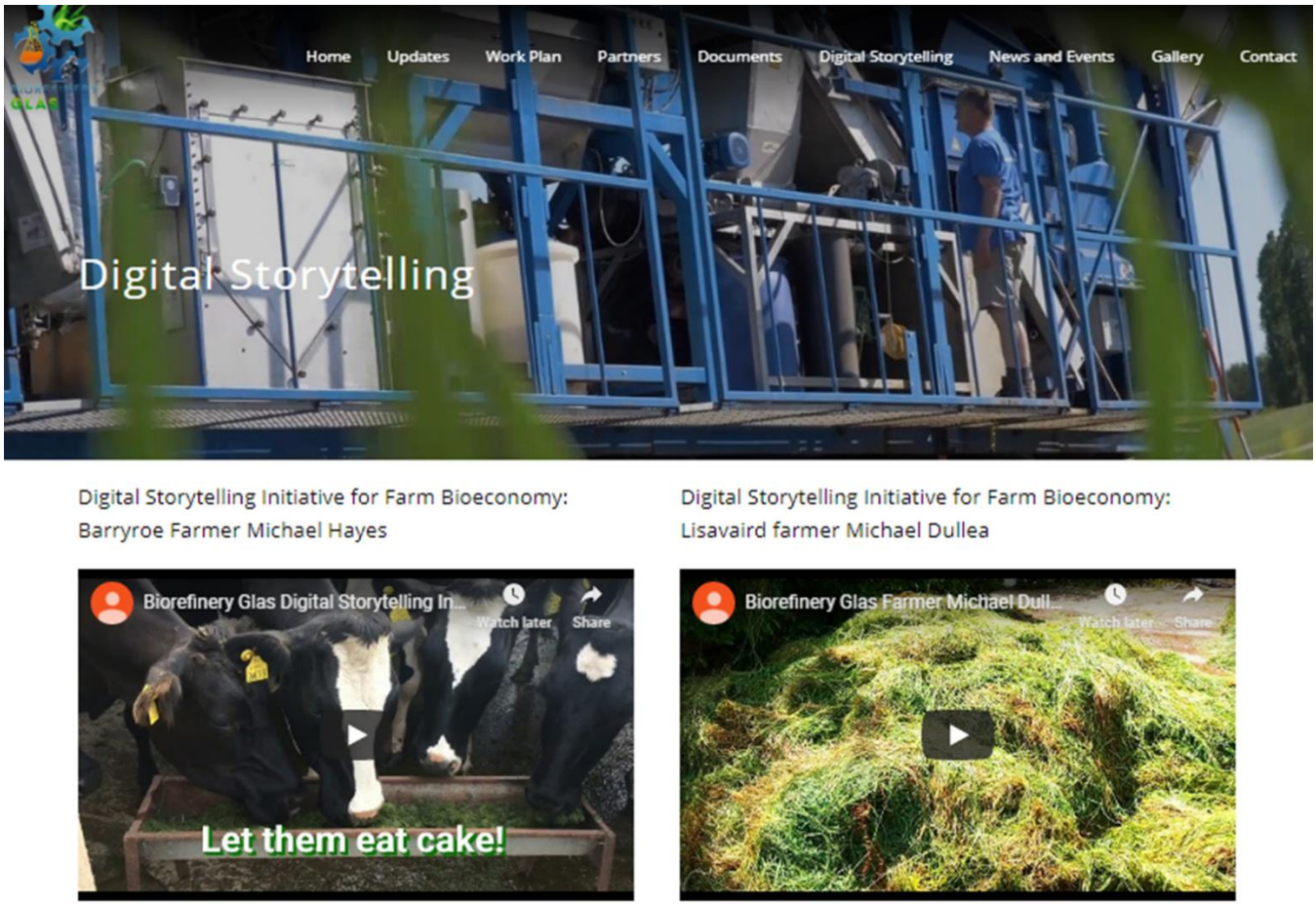

Figure 17: Biorefinery Glas Digital Storytelling Initiative[38]

7.4 Collaborative Dissemination

In order to build capacity, Biorefinery have collaborated on dissemination activities including co-hosting of events and co-dissemination (e.g. newsletter collaboration) with a variety of relevant national and EU projects as indicated below:

- EIP-Agri Small-scale Biogas Demonstration – hosting workshop[39]
- Power4Bio – Newsletter Co-Dissemination[40]
- Enabling Project – Newsletter Co-Dissemination[41]
- BioVoices – Newsletter Co-Dissemination
- Rubizmo – Dissemination[42]

[38] https://biorefineryglas.eu/digital-storytelling/
[39] https://www.irbea.org/farmbiogas/
[40] https://power4bio.eu/featured-project-biorefinery-glas-a-farmer-centric-bioeconomy-approach
[41] https://www.enabling-project.com/news-1/2019/5/8/eip-agri-operational-group-kick-off-irelands-first-biorefinery-project-with-carbery-group-and-barryroe-agri-co-op
[42] https://rubizmo.eu/news/view/078d5399-c7af-463d-a678-e501460dd276

- Grassification[43]

Biorefinery Glas is also a member of the European Bioeconomy Network[44], an EU initiative aimed at supporting dissemination of bioeconomy projects and their events.

7.5 Awards

Biorefinery Glas was a recipient of the RDS Spring Awards in the category of Sustainable Rural Innovation (1st Runner Up). In December 2020, Biorefinery Glas participants from Carbery, UCD and MTU met with the RDS award team to shoot a video about the project which was aired during the award ceremony[45].

7.6 Peer Reviewed Publications

Since the end of the project, Biorefinery Glas team members have proceeded to convert some of the project results into peer-reviewed scientific publications. To date three publications in peer-reviewed journals have been produced while another two are in progress (see appendix).

7.7 Farmer Workshops

From the early stages of the project, particularly in the run up to demonstration phase, participating farmers were included in project meetings in order to ensure that all logistical and technical considerations could be planned in advance of the demonstrations. In addition to technical workshops, a workshop to train farmers on the procedures for digital storytelling activities was delivered. This equipped farmers with the skills required to produce a video of their experience during the biorefinery and feeding trial activities.

A demonstration day (described below) and two results seminars were hosted specifically to communicate the results of the project to participating and external farmers. The two workshops focused on farmers were closed workshops which were only available to farmers, while a separate webinar-based final conference was hosted to present results to the broader public stakeholders. While initially these events were planned as in-person events, due to COVID-19 restrictions, it was necessary to move these online via zoom. These workshops took place on the final week of February 2021 (22nd, 23rd and 24th).

In addition to digital stories and project deliverables, partners have also developed fact sheets available through the project website which provide farmers with a shorter synopsis of the different findings from project activities.

7.8 Demonstration Day

In order to improve visibility of the grass biorefinery the Biorefinery Glas Operational Group hosted a demonstration to attract external farmers and other stakeholders to visit the biorefinery in operation. The demonstration day was hosted on 11th July 2019 at Shinagh Estates Farm in Cork. The demonstration was a chance for farmers to see the machine in operation at close quarters. The OG hosted the event in the form of a farm walk, consisting of various stops and talks from OG participants

[43] https://www.biorefine.eu/projects/grassification
[44] https://eubionet.eu/partners/
[45] https://www.youtube.com/watch?v=tGID8mVn8qI&t=12s

in Shinagh Farm on relevant aspects of the projects. The following boards were included in the farm walk:

1. Welcome and introduction to the Bioeconomy including Carbery Biorefinery in Balineen
2. Introduction to grass biorefinery and GRASSA process used in Biorefinery Glas
3. Overview of products being produced in the project and how the products will be tested
4. Overview of sustainability aspects including zero-waste approach, potential for renewable energy generation and improvements in nitrogen-use-efficiency
5. An overview of ongoing research at Shinagh Farm

Around 300 stakeholders, primarily farmers participated on the day. Information packs were provided to participants, containing key project information and contacts. The demonstration day was advertised and featured in local and national farming newspapers including the Irish Farmers Journal[46] and the Southern Star. The OG collaborated with Teagasc on the design and promotion of the event, and it was recognised as a DAFM-approved KT event for Beef and Dairy farmers.

Figure 18: Introducing farmers to green biorefinery process and products during the demonstration day

[46] https://www.farmersjournal.ie/watch-open-day-at-west-cork-grass-biorefinery-project-477191

Figure 19: Photos of various stops during the demonstration day

8.0 Appendix – Publications and Materials

During the course of the project Biorefinery Glas have produced a variety of public reports and project materials which are accessible through the project website documents section[47]. Publications available include:

- Public Deliverable 1.3: Operational Guide for Grass Biorefinery
- Public Deliverable 2.1: Report on potential of press cake as a fodder source for dairy cows (executive summary available online, full deliverable will upload once peer-reviewed publication has been approved)
- Public Deliverable 2.4: Report on potential of grass protein juice concentrate as soybean replacement in pig feed
- Public Deliverable 2.5: Report on prebiotic potential of fructo-oligosaccharides in grass
- Public Deliverable 2.6: Report on potential of recirculated grass biorefinery whey as nutrient fertiliser and opportunities for grass whey and presscake in biogas production
- Consortium Deliverable 3.1: Executive summary available online (full version submitted to DAFM)
- Public Deliverable 3.2: Guide on Financing Options for Farmer Uptake of Small-Scale Biorefinery
- Public Deliverable 3.3.: Policy recommendations to enable farmer-led development of small-scale grass biorefineries
- Public Deliverable 3.4: Pre-commercial product specifications and regulatory compliance action plans
- 5 Fact Sheets
- Practice Abstract
- Results Presentation
- Brochure
- Infographics
- Peer-reviewed publications include (texts included within appendix):
    - Serra, E., Lynch, M.B., Gaffey, J., Sanders, J.P.M., Koopmans, S., Markiewicz-Keszycka, M., Bock, M.H., McKay, Z.C. and Pierce, K.M., 2023. Biorefined press cake silage as feed source for dairy cows: Effect on milk production and composition, rumen fermentation, nitrogen and phosphorus excretion and in vitro methane production. Livestock Science, 267, p.105135.
    - Ravindran, R., Koopmans, S., Sanders, J.P., McMahon, H. and Gaffey, J., 2021. Production of green biorefinery protein concentrate derived from perennial ryegrass as an alternative feed for pigs. Clean Technologies, 3(3), pp.656-669.
    - Ravindran, R., Donkor, K., Gottumukkala, L., Menon, A., Guneratnam, A.J., McMahon, H., Koopmans, S., Sanders, J.P. and Gaffey, J., 2022. Biogas, biomethane and digestate potential of by-products from green biorefinery systems. Clean Technologies, 4(1), pp.35-50.

[47] https://biorefineryglas.eu/documents/

Figure 20: Sample deliverables and downloads

Figure 21: Project Brochure


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eip-agri

Ireland's European Structural and Investment Funds Programmes 2014-2020
Co-funded by the Irish Government and the European Union

Department of Agriculture, Food and the Marine
An Roinn Talmhaíochta, Bia agus Mara

The European Agricultural Fund for Rural Development: Europe investing in rural areas


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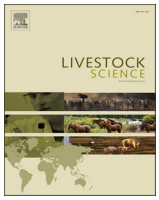

Biorefined press cake silage as feed source for dairy cows: effect on milk production and composition, rumen fermentation, nitrogen and phosphorus excretion and *in vitro* methane production

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HIGHLIGHTS

- Press cake silage can partially replace grass silage in the diet of dairy cows.
- Press cake group maintained similar milk production to the grass silage only group.
- Experimental treatment improved nitrogen use efficiency.
- In the *in vitro* study the methane production was not affected by treatment.

ARTICLE INFO

Keywords:
Biorefinery
Dairy cow
Nitrogen
Phosphorus

ABSTRACT

The objective of this study is to investigate the effect of replacing grass silage with biorefined grass silage (press cake silage) on dry matter intake (DMI), milk production and composition, rumen fermentation parameters, nitrogen and phosphorus excretion of early lactation Holstein Friesian dairy cows. An *in vitro* experiment using the rumen simulation technique (RUSITEC) also investigated the *in vitro* dry matter disappearance and methane (CH_4) production of these feedstuffs. In this study, press cake silage was made from perennial ryegrass (*Lolium perenne*) using a novel biorefining process. Thirty early-lactation cows (*Bos taurus* strain Holstein Friesian) were used in a randomized complete block design experiment ($n = 15$) and offered two dietary treatments for a 56 d period: Grass silage (GS): 14 kg dry matter (DM) grass silage + 7.2 kg DM of concentrate + 0.44 kg DM of soyabean meal; Press cake (PC): 5 kg DM grass silage + 9 kg DM press cake silage + 7.2 kg DM concentrate + 0.44 kg DM soyabean meal. The dietary treatments were also incubated *in vitro* for a period of 18 days using the RUSITEC. In the *in vivo* study, DMI was lower for PC compared to GS. No difference was observed between the treatments for milk yield and milk quality; however, milk fat yield was lower and milk solids yield tended to be lower in PC compared to GS. Cows offered PC had higher N use efficiency (NUE, milk N/N intake), lower total N excretion and lower N excretion in feces and urine compared to cows offered GS. Total and fecal P excretion was lower in cows fed PC compared to cows fed GS. Ruminal NH_3-N concentration was lower when PC was offered. *In vitro* rumen fermentation parameters such as pH, volatile fatty acids and CH_4 output were not affected by treatment. *In vitro* dry matter disappearance and NH_3-N concentration were lower for PC compared to GS. This study suggests that press cake silage can partially replace grass silage in the diet of dairy cows with beneficial effects on the environment and without compromising animal productivity.

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1. Introduction

Environmental sustainability and protein source availability are key challenges for the agriculture sector in Europe (European Environmental Agency EEA, 2012). The need to increase resource efficiency while reducing the use of imported protein sources has led to the development of 'green biorefinery', which primarily involves the processing of fresh grass or silage into a variety of new products, offering the potential to improve the efficiency of protein use and the sustainability of grassland (Kamm et al., 2016; Ravindran et al., 2021).

In Ireland, grassland is the dominant biomass resource, accounting for approximately 90% of the agricultural land area (CSO, 2020). Compared to other European countries, Ireland has a climate that is well suited to growing grass (Läpple et al., 2012) and farms have the potential to produce between 12 and 16 tonnes of grass DM per hectare annually (O'Donovan et al., 2011; Byrne et al., 2017). However, the efficiency of grass utilization on Irish dairy farms is still relatively low, estimated to be about 8 tonnes of grass DM per hectare (Läpple et al., 2012; Tubritt et al., 2019). Therefore, there is a potential to increase the utilisation of this resource, possibly through the production of a range of products from freshly harvested grass.

In most of the green biorefinery processes, the first step is the mechanical liquid-solid separation of the cell content (press juice) from the plant structural framework (press cake). These fractions can be further processed to recover or produce valuable products (McEniry and O'Kiely, 2013; Franco et al., 2019; Ravindran et al., 2021). The press juice contains soluble proteins, free amino acids, sugars, organic acids, dyes, enzymes, hormones, further organic substances, and minerals (Xiu and Shahbazi, 2015). A protein rich concentrate can be precipitated and dried from the press juice resulting in a protein supplement that can be fed to non-ruminant animals, such as pigs (Damborg et al., 2019; Franco et al., 2019; Ravindran et al., 2021). Press cake, which is the solid fraction that remains after the biorefinery process, has been identified as a potentially valuable product in ruminant diets due to its high fiber and insoluble protein content (Savonen et al., 2018; Damborg et al., 2018). In addition, press cake is lower in N and P compared to grass silage and its inclusion in the diet of dairy cows has the potential to reduce N and P excretion (Pijlman et al., 2018).

A study by Damborg et al. (2019) observed an increase in milk yield, milk fat (kg/d), milk protein (kg/d) and lactose (kg/d) when feeding grass-clover press cake silage, made from fresh grass-clover, compared to unrefined grass-clover silage. Savonen et al. (2020) reported that feeding press cake silage, made from silage, when compared with unrefined silage did not affect milk yield, milk fat and lactose yields, however, milk protein yield decreased with the increase of press cake in the diet. In the same study, rumen fermentation parameters were not affected and an improvement in nitrogen use efficiency (NUE) was observed. However, there is still a significant gap in the knowledge regarding the use of press cake silage as a feedstuff on milk production, milk composition, rumen fermentation and methane (CH_4) production where the feed source is predominantly perennial ryegrass.

The objective of this study was to investigate the effect of partial replacing of grass silage with biorefined press cake silage on DMI, milk production and composition, rumen fermentation, N and P excretion of early lactation Holstein Friesian dairy cows and on *in vitro* dry matter disappearance and CH_4 production using the rumen simulation technique (RUSITEC). It was hypothesized that replacing grass silage with biorefined press cake silage would not affect milk production, milk composition and rumen function and would result in a reduction in N and P excretion.

2. Materials and methods

2.1. Experimental feed

The production of press cake silage was part of the Biorefinery Glas project (Biorefinery Glas -Small-scale Farmer-led Green Biorefineries) and involved five farms across the Carbery Cooperative region (Carbery, Ballineen, Co. Cork, Ireland). The grass, predominantly perennial ryegrass (*Lolium perenne)* with 3% of unsown species, was harvested twice daily using a zero grazer (ZG75 Zero Grazer Dromone, Oldcastle, Co Meath, Ireland) and transported to the biorefinery machine, supplied and operated by GRASSA BV (Venlo, Limburg, The Netherlands). The grass use for press cake silage was harvested and processed between July 1st to August 7th 2019. During this process, the harvested grass was squeezed through a twin screw press to separate the juice fraction from the fiber fraction. At the end of the harvest, the press cake silage bales were transported to University College Dublin (UCD) Lyons Farm, (Lyons Estate, Celbridge, Naas, Ireland, 53°17′56″ N, 6°32′18″ W), stored and later used for the animal feeding study.

The control grass silage, predominantly perennial ryegrass (*Lolium perenne*) with 3% of unsown species, was produced at UCD Lyons Farm. Grass was mowed on the 14th of May 2019 and wilted for 24 h in the field before being chopped to a 40 mm size and ensiled in a pit on the 15th of May (without additives). Chemical composition of the press cake silage and control silage are presented in Table 1.

2.2. In vivo experiment

2.2.1. Animal ethics

The Animal Research Ethics Committee at UCD approved all the procedures described in this experiment. These procedures were conducted under experimental license (AI18982/P169) from the Health Products Regulatory Authority (HPRA) under European directive 2010/63/EU and S.I. No. 543 of 2012. All procedures carried out during this experiment were classified as mild in severity banding. Hence, no pain, distress or suffering was experienced by the cows involved in this experiment and no humane endpoints were established. Each person who carried out procedures on experimental animals during the course of this experiment was licensed to do so by means of Individual Authorisation from the HPRA.

2.2.2. Cows, treatment and experimental design

Twenty-four multiparous and six primiparous early-lactation dairy

Table 1
Chemical composition (g/kg) of diets and ingredients.

	Diets		Experimental feedstuffs			
Chemical composition (g/kg DM unless stated)	GS [1]	PC [2]	Grass silage	Press cake silage	Standard concentrate	Soyabean meal
DM	411.8	486.3	299.4	374.1	900	880
Ash	100.4	67.5	98.3	42.0	69.2	86.6
Crude Protein	180.2	152.2	164.4	109.3	188.2	532.5
NDF [3]	373.3	569.4	490.9	740.9	153.5	75.4
ADF [4]	225.2	270.7	289.7	413.3	78.6	41.6
WSC [5]	46.2	42.0	42.6	35.6	NA [6]	NA
Starch	109.7	108.6	15.5	13.3	486.0	25.8
Phosphorus	4.6	4.3	4.2	3.6	6.1	7.0
AIA [7]	20.8	11.3	27.4	4.3	9.6	4.0
Ether extract	46.8	25.5	34.6	28.2	25.2	12.9
Gross energy (MJ/kg of DM)	17.98	18.15	17.65	18.30	17.70	19.60

[1]Grass silage treatment (14 kg DM of grass silage + 7.2 kg DM standard concentrate +0.44 kg DM soyabean meal).
[2]Press cake treatment (5 kg DM grass silage + 9 kg DM press cake silage + 7.2 kg DM standard concentrate + 0.44 kg DM soyabean meal).
[3]Neutral Detergent Fibre (NDF).
[4]Acid Detergent Fibre (ADF).
[5]Water Soluble Carbohydrate (WSC).
[6]Not analyzed (NA).
[7]Acid Insoluble Ash (AIA).

cows (*Bos taurus* strain Holstein Friesian) were selected from the autumn-calving dairy herd at UCD Lyons Farm. The cows were blocked on parity and balanced for DIM, milk yield, milk composition (fat %, protein %, fat and protein kilogram) and BCS, to help reduce bias (mean ± SD: 2.6 ± 1.6 parity; 69 ± 3.6 DIM; 1157.3 ± 279.7 milk yield; 4.3 ± 0.5 fat %; 3.7 ± 0.2 protein %; 49.5 ± 12.4 fat kg; 42.7 ±9.5 protein kg; 2.8 ± 0.3). The primiparous cows were also balanced for BW (mean ± SD: 531 ± 21.2). Cows were assigned to one of two dietary treatments in a randomized complete block design ($n = 15$). Each cow was considered an experimental unit. A power test analysis was conducted to estimate the sample sizes using the coefficient of variation of milk fat yield (Clark et al., 2009; Alzahal et al., 2010). Blinding was ensured as the author was aware of group allocation during allocation but not during the experiment or the data analysis. The experiment was conducted between December 2019 and February 2020 and ran for a total of 70 days (d), including a 14 d dietary acclimatization period and a 56 d experimental period. For the entire duration of the experiment cows were housed in a free stall barn, bedded with hydrated lime and sawdust, with *ad libitum* access to feed and water.

The two dietary treatments were as follows: Grass silage (GS): 14 kg DM of grass silage plus 7.2 kg DM of concentrate and 0.44 kg DM of soyabean meal; Press cake (PC): 5 kg DM of grass silage, 9 kg DM of press cake silage, 7.2 kg DM of concentrate and 0.44 kg DM of soyabean meal. Both dietary treatments were offered as partial mixed ration (PMR) and fed in the morning via a Keenan (Borris, Co. Carlow, IE) diet feeder into computerized feeding stations (RIC System, Insentec B.V., Marknesse, NE). Every day press cake silage offered was a mixture of bales out of the 5 farms to maintain a constant nutritional value over the study period. Half of the concentrate allowance was offered in the parlour at milking twice daily, while the remaining concentrate was offered in the PMR.

2.2.3. Data and sample collection

Feed collection. Samples of the PMR were taken daily and pooled weekly, single ingredient samples were taken once a week and pooled for the duration of the trial for each dietary treatment. These samples were then stored for analysis: DM, ash, gross energy, ether extract, CP, NDF, ADF, water-soluble carbohydrates (WSC), starch, acid insoluble ash (AIA; Table 1) and apparent total tract dry matter digestibility (DMD; Table 2). Dry matter intakes of the PMR were calculated from the computerized feeding stations (RIC System, Insentec B.V., Marknesse, NE). Half of concentrate allowance was entirely consumed in the milking parlour and was added to the PMR intake to calculate total DMI.

Cow measurements. Cows were milked twice daily at 0700 and 1500 h. Milk yield measurement and milk sample collection were carried out using the Weighall milk meter system (Dairymaster, Causeway, Kerry, IE). Milk samples were taken once a week during consecutive evening and morning milking's and pooled on a per cow basis in proportion to evening and morning yield.

Cows were weighed twice daily using an electronic scale as they exited the milking parlour, the measurements were averaged on a daily basis. Body condition score was determined once a week, by the same trained person, using a scale of 1 to 5 with 0.25 increments according to Edmonson et al. (1989).

Blood samples were collected once a week by jugular venepuncture after evening milking. Blood samples for glucose analysis were harvested into a 4 mL Vacutainer tube containing potassium oxalate and sodium fluoride (Ref. 3668,201; BD Plymouth, PL6 7BP, UK) and centrifuged at 2100 × g for 20 min at 4°C for extraction of blood plasma. The blood plasma was then drained and frozen at -20°C pending analysis. Blood samples for non-esterified fatty acids (NEFA) and BHB analysis were harvested into a 10 mL Vacutainer tube coated in silicone (Ref. 367,896; BD Plymouth, PL6 7BP, UK) and allowed to clot for 24h at 4°C before centrifuging at 1900 × g for 20 min at 4°C for extraction of serum. Samples were frozen at -20°C pending analysis.

Rumen fluid samples were collected after evening milking once a week using the Flora Rumen Scoop oesophageal sampler (Prof-Products, Guelph, ON, Canada) each week, to coincide with milk, blood sampling and BCS assessment. Rumen fluid samples were immediately analysed for pH (Phoenix Instrument EC-25 pH/Conductivity Portable Meter) and then strained through 4 layers of cheesecloth. A 4 mL aliquot was mixed with 1 mL of 50% trichloroacetic acid and stored at -20°C pending analysis for volatile fatty acids (VFA) and NH_3-N concentration.

Nitrogen and phosphorus partitioning study. A N and P partitioning study was conducted on week 6 of the experimental period. Samples of PMR, concentrate, milk, feces, and urine were collected during this period (Whelan et al., 2017; McKay et al., 2019). Samples of the diets were taken daily for a duration of 5 d and pooled per dietary treatment. Daily DMI were calculated from the computerized feeding stations. Milk samples were collected daily during am and pm milking, pooled on a per cow basis according to milk yield and sent for analysis to a commercial milk laboratory. For five consecutive d following pm milking, fecal samples were collected during the observation period as cows naturally defecate and if not, samples were collected per rectum and placed in a forced air oven at 55°C until dry. Dried feces were later pooled per cow for analysis. Urine samples were collected following gentle massaging of the area directly above the mammary gland at the rear of the cow for two d after pm milking. Urine samples were acidified with 75% H_2SO_4 to prevent NH_3 volatilization and an aliquot was stored at -20°C pending

Table 2
The effect of treatment on dry matter intake, feed efficiency, body condition score, body weight, milk production and milk composition.

Item	Treatment		SEM	*P*-value
	GS [1]	PC [2]		
DMI				
PMR [3] (kg DM/d)	15.73	14.40	0.342	0.01
Total (kg DM/d)	19.33	18.00	0.342	0.01
Feed efficiency [4]	1.31	1.27	0.024	0.24
DMD (%) [5]	70.75	72.00	0.871	0.31
Start BCS	2.97	2.93	0.069	0.74
End BCS	2.91	2.86	0.058	0.55
BCS change	-0.05	-0.06	0.053	0.83
Start BW (kg)	651.63	647.8	21.095	0.89
End BW (kg)	663.17	654.33	19.183	0.74
BW change (kg)	11.53	6.53	14.522	0.80
Milk production (kg/d)				
Milk yield	28.02	27.33	0.724	0.51
Fat	1.28	1.18	0.031	0.03
Protein	0.97	0.94	0.019	0.34
Milk solids	2.24	2.11	0.046	0.05
Lactose	1.23	1.21	0.027	0.71
Casein	0.75	0.74	0.015	0.41
Milk composition %				
Fat	4.58	4.35	0.133	0.24
Protein	3.47	3.44	0.071	0.79
Lactose	4.47	4.49	0.014	0.35
Casein	2.79	2.76	0.055	0.72
Urea (g/100g of milk)	0.027	0.024	0.0007	0.01
SCC (x 10^3 cells/mL) [6]	27	29	3.613	0.06
ECM (kg) [7]	24.94	23.33	0.044	0.04

[1]Grass silage treatment (14 kg DM of grass silage + 7.2 kg DM standard concentrate +0.44 kg DM soyabean meal).
[2]Press cake treatment (5 kg DM grass silage + 9 kg DM press cake silage + 7.2 kg DM standard concentrate + 0.44 kg DM soyabean meal).
[3]Partial mixed ration.
[4]Feed efficiency = kg of ECM/kg of DMI.
[5]Apparent total tract dry matter digestibility (DMD).
[6]For SCC, data was transformed by conducting a Box-Cox transformation analysis to calculate *P*-value. The corresponding least squares means and standard errors of the non-transformed data are presented in results for clarity.
[7]Energy Corrected Milk (ECM) = [(0.03273 × milk yield kg) + (7.65 × milk protein kg) + (12.97 × milk fat kg)].

analysis.

Sample analysis. Single ingredient samples (silage, concentrate and soyabean meal), PMR and fecal samples were dried in a forced air oven at 55°C for 48 h and were ground in a hammer mill fitted with a 1 mm screen (Lab Mill, Christy Turner. Ltd., Ipswich, UK). The DM content of the samples was determined after drying for 16 h at 105°C (AOAC International, 2005a, method 960.15). The ash content was determined following combustion in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany) at 550°C for 5.5 h (AOAC International, 2005b, method 942.05). The N content of single ingredients, PMR and fecal samples was determined by combusting using and CP content was calculated using N × 6.25 (FP 828p Analyzer, Leco Corp, St Joseph, Michigan, US; AOAC International, 2005c, 990.03). The NDF and ADF content was determined according to the method of Van Soest et al. (1991) using the Ankom 220 Fibre Analyzer (ANKOM Technology 2052 O'Neil Road, Macedon NY 14502). Partial mixed ration samples, concentrate and soyabean meal samples were analysed by adding 4mL of thermostable α-amylase and 20g of sodium sulphite (Na_2SO_3), whereas silage samples were analysed with neutral detergent solution only. Starch content was determined on PMR, concentrate and soyabean meal samples using the Megazyme Total Starch Assay Procedure (product no: K-TSTA; Megazyme International Ireland Ltd, Wicklow, IE). Gross energy content of concentrate, soyabean meal and PMR samples was determined by bomb calorimeter (Parr 1281 Bomb Calorimeter, Parr Instrument Company, Moline, IL). The ether extract was determined using Soxtex instrument (Tecator, Hoganas, Sweden) and light petroleum ether. The concentration of WSC was determined on silage samples as described by Dubois et al. (1956).

The AIA was determined according to the European Commission (2009) using 2N HCl on grass silage, press cake silage, concentrate, soyabean meal, and fecal samples. The DMD was determined using AIA as an internal marker (Van Keulen and Young, 1977) as: DMD = 100 × ((1/Mfeed) - (1/Mfeces))/(1/Mfeed), where Mfeed = AIA concentration in the feed and Mfeces = AIA concentration in the feces. The DMI used to calculate DMD refers to the data collected during week 6 of the experimental period.

Milk samples were sent to a commercial milk laboratory (National Milk Laboratories Ltd, Unit 26 - 29, Laches Close, Calibre Industry Park, Four Ashes, Wolverhampton, UK, WV10 7DZ) for determination of milk fat, protein, lactose, casein, urea, and SCC concentration using mid-infrared spectrometry (Milkoscan FT6000, FOSS, 2005; Soyeurt et al., 2006). Values for energy corrected milk (ECM) were calculated as follow: ECM = [(0.03273 × milk yield kg) + (7.65 × milk protein kg) + (12.97 × milk fat kg)] (Tyrrell and Reid, 1965)

Blood samples were analyzed for NEFA (Kit No. FA115) and BHB (Kit No. RB1007) using enzymatic tests. Glucose (Kit No. GL3816) was analyzed using the hexokinase test. All kits were sourced from Randox Laboratories Ltd (Crumlin, County Antrim, UK). All blood analyses were carried out using a clinical blood analyzer (RX imola; Randox Laboratories Ltd) in the UCD veterinary clinical pathology laboratory (School of Veterinary Medicine, UCD, Belfield, Dublin, Ireland).

Rumen fluid samples were allowed to thaw overnight at 4°C and centrifuged at 1800 × g for 10 min at 4°C. One ml of supernatant was diluted with 4 mL of distilled water and then centrifuged at 1800 × g for 15 min at 4°C using the phenol-hypochlorite method of Weatherburn (1967). The NH_3-N concentration was measured using a spectrophotometer (Shimadzu UK Ltd, Wolverton Mill South, Milton Keynes, UK). Rumen fluid samples were also prepared for VFA analysis by mixing 250 μL of the same supernatant used for NH_3 determination with 3.75 mL of distilled water and 1mL of internal standard solution (0.5 g 3-methyvaleric acid in 1,000 mL of 0.15 M oxalic acid). The resulting solution was centrifuged at 260 × g for 5 min at room temperature and then filtered through a syringe tip filter (polytetrafluoroethylene, 25 mm diameter, 0.45 μm) into 2 mL gas chromatography vials. Concentration of VFA's was determined using Scion 456-GS (Scion Instrument, Scotland, UK) fitted with a DB-FFAP capillary column (15 m × 0.53 mm: 1.00 μm, Agilent Technologies, USA).

Individual cows DMI of PMR was calculated from the computerized feeding stations. Total DMI was calculated adding the PMR intake and the concentrate consumed in the milking parlour. The apparent digestibility coefficient of nutrients (ND) was determined as: ND = 100 × (Nfeed/Mfeed) - (Nfeces/Mfeces)/(Nfeed/Mfeed) where Mfeed = AIA concentration in the feed; Mfeces = AIA concentration in the feces; Nfeed = nutrient concentration in the feed; Nfeces = nutrient concentration in the feces. Feces N output was calculated as: Feces N output = (1 – N digestibility) × N intake

Urine N output was estimated as follow: Urine N output = N intake – milk N yield – feces N output assuming that N retention was zero.

The P content was determined on PMR, concentrate, feces, urine and milk following the ISO 6491:1998 method. The residual ash of PMR, concentrate, feces and milk samples was solubilized in 20% HCl HNO_3. The resulting solutions were filtered through filter paper (22 μm porosity) and then diluted 1 in 25 (PMR and concentrate), 1 in 2 (milk) and 1 in 50 (feces) with distilled water. Five millilitres of the diluted sample were then combined with 5 ml of molybdovanadate reagent, allowed to stand for 15 min and then analysed for P concentration using a spectrophotometer (Shimadzu UK Ltd, Wolverton Mill South, Milton Keynes, UK).

The P concentration in PMR, concentrate, milk and feces was calculated as follow: P %= (ppm sample × 100 x dilution factor) / 10 × sample weight × 1000 where ppm sample = (10 × absorbance sample) / absorbance standard.

The P concentration in urine was determined by adding 2 mL of the acidified urine, 10 mL of molybdate I solution and 4 mL of 0.25% aminonaphthol-sulfuric acid into 84 mL of distilled water (Fiske and Subbarow, 1925). The solution was incubated for 5 min in a water bath at 37°C and then analysed using a spectrophotometer (Shimadzu UK Ltd, Wolverton Mill South, Milton Keynes, UK).

The P concentration in urine was calculated as followed: P % = (absorbance sample / absorbance standard) × 0.4

2.3. *In vitro study*

2.3.1. *Apparatus and experimental design*

This experiment was conducted using a single six-vessel RUSITEC system (Sanshin Industrial Co. Ltd, Yokohama, Japan) to simulate the rumen environment *in vitro* during an 18d period. The incubation procedure described by Czerkawski and Breckenridge (1977) was followed throughout the experiment. The same dietary treatments offered in the *in vivo* experiment (described above) were used. These were incubated in the same inoculum, replicated three times and distributed randomly in the RUSITEC apparatus (n = 3). Each vessel was considered an experimental unit. Blinding was ensured as the author was aware of vessel allocation during allocation but not during the experiment or the data analysis.

All vessels were fed 15.5 g DM of feed components. The GS treatment consisted of 10 g of grass silage, 5.2 g of concentrate and 0.3 g of soyabean meal; PC treatment consisted of 3.4 g of grass silage, 6.6 g of biorefined press cake silage, 5.2 g of concentrate and 0.3 g of soyabean meal. Prior to inclusion in the vessels, concentrate and silage components were dried in a forced air oven at 55°C for 48 h; concentrate component was ground in a hammer mill fitted with a 1 mm screen and the silage component (grass silage and press cake silage) was not ground.

2.3.2. *Experimental procedure*

On the first d of the experiment, rumen fluid and solid digesta were collected before milking time at 0730h from three lactating rumen-fistulated Holstein Friesian dairy cows (experimental license (AI18982/P131) from the HPRA under the European directive 2010/

63/EU and S.I. No. 543 of 2012). The rumen inoculum was strained through four layers of cheesecloth, flushed with CO_2 and transferred to the RUSITEC vessels within 30 minutes from collection. Each vessel was inoculated with 450 mL of rumen inoculum and 350 mL of artificial saliva (McDougall, 1948) and kept in a water bath at 39°C. Dietary treatments were added to each vessel in nylon bags (ANKOM *in situ* forage and concentrate bag 50 μm porosity; R1020). A bag containing 70 g of solid digesta, a second bag containing 5.5 g of concentrate mix (concentrate and soyabean meal) and a third bag containing 10 g of silage treatment of interest (GS or PC) were placed in the feed container in each vessel. This container was immersed in the vessel liquid, the vessel was then closed and returned to the water bath. The vessel was connected to a plunger and the motor engaged. Artificial saliva was prepared daily and was constantly infused at a rate of 26 mL/h using a peristaltic pump. The displaced effluent (overflow) and fermentation gases from each vessel were collected into effluent bottles and gas collection bags, respectively. Overflow bottles were kept in a water bath at 2°C to stabilise fermentation products and gas bags were reusable polyethylene bags fitted with one-way valves. After 24 h each vessel was opened, bags containing rumen solid digesta and concentrate were removed, washed, and squeezed in 50 mL of artificial saliva. The liquid fraction resulting from the washing was returned to the vessel and two new nylon bags, containing the silage and the concentrate treatment, were inserted into the feed container. On subsequent d, silage bags were removed after 48 h and replaced with new bags of the same silage, while concentrate bags were replaced after 24 h.

2.3.3. Data collection

Days 0-10 of the experiment allowed for microbial acclimatization and fermentation stabilisation; on d 11-17 samples were collected. For the entire duration of the experiment, the pH of the vessel and overflow liquid, quantities of the overflow liquid and gas volumes were recorded. On d 11-17 liquid samples were collected from the vessel and overflow containers for analysis of rumen fermentation parameters (NH_3 and VFA's concentration). On d 15 and 16 vessels were sampled at 0h, 2h, 4h, 6h, 8h, 12h and 24h after treatment incubation. The pH of the liquid samples was measured immediately using a digital pH meter (Phoenix Instrument EC-25 pH/Conductivity Portable Meter). A 4 ml sample was then preserved using 1mL of 50% TCA and frozen (-20°C) pending analysis. Nylon bags were collected daily, rinsed in iced water and stored at -20°C for determination of DM disappearance.

On d 11-17 total gas production was measured using a DC dry gas test meter (Shinagawa Corp.; Tokyo, Japan), and CH_4 concentration was analysed using an infra-red gas analyser (GC100 portable CH_4 reader, ADC Gas Analysis; Hoddeston, UK).

2.3.4. Chemical analysis

Vessel liquid and overflow samples were analysed for NH_3 and VFA concentration as described above. The feed residues were allowed to thaw gradually at 4°C, then washed in a domestic washing machine using the cold rinse cycle in the absence of detergent (30 min) to remove the bacteria attached loosely to the bags. The feed residues were then dried in a 55°C forced air oven for 48 h and weighed. Feed DM disappearance was calculated as the amount of material that had disappeared from the nylon bags after 24 h and 48 h of incubation, for concentrates and silage, respectively.

2.4. Statistical analysis

Data was checked for normality and homogeneity of variance by histograms, qq plots, and formal statistical tests as part of the UNIVARIATE procedure of SAS (version 9.4; SAS Institute, 2018). Data that was not normally distributed was transformed by raising the variable to the power of lambda. The appropriate lambda value was obtained by conducting a Box-Cox transformation analysis using the TRANSREG procedure of SAS (Fahey et al., 2007). Somatic cell count required transformation and was raised to the power of -0.5. The transformed data were used to calculate the *P*-values. However, the corresponding least square means and standard errors of the untransformed data are presented in results. Analysis of the *in vivo* data was conducted using a mixed model ANOVA (PROC MIXED). Each cow was considered as experimental unit for all parameters. The model included the fixed effect of treatment (GS and PC), day, BW, DMI, DIM and their interactions. Interactions were removed from fixed effects as $P > 0.10$. Random effects (cow) and repeated measures (d) were included in the model.

In vitro data were analysed using a mixed model ANOVA (PROC MIXED). Each vessel was considered as experimental unit for all parameters. The model included the fixed effect of treatment, d and their interaction. Interactions were removed from fixed effects where $P > 0.10$. Fermentation vessels were included as a random effect and d was included as repeated measures. A simple and heterogeneous first order autoregressive covariance structure was used based on the smallest Bayesian information criterion value. Statistically significant differences between least squares means were tested using the PDIFF command for pairwise comparisons of treatment means. Statistically significant difference was assumed at $P < 0.05$ and a tendency toward significance was assumed at $P \geq 0.05$ but $P < 0.10$.

3. Results

3.1. Chemical composition of experimental feeds

Table 1 shows the chemical composition of the single ingredients and dietary treatments. The main differences between the two dietary treatments are the DM content (411.8 g/kg for the GS, 486.3 g/kg for PC), the CP concentration (180.2 g/kg of DM in GS, 152.2 g/kg of DM in PC), the concentration of NDF (373.3 g/kg of DM for GS, 569.4 g/kg of DM for PC), the P content (4.6 g/kg of DM in GS, 4.3 g/kg of DM in PC), and the DMD (70.76% for GS and 72.01% for PC).

3.2. In vivo experiment: DMI, feed efficiency, BCS, BW, milk production, milk composition, rumen fermentation and blood metabolites

Table 2 shows the effect of partial replacement of grass silage with biorefined press cake silage on DMI, BCS, BW, milk production and milk composition. Cows offered PC had a lower ($P = 0.01$) PMR intake and total DMI than GS. Feed efficiency (kg of ECM/kg of DMI) was not affected by treatment; however, cows offered PC produced less ($P = 0.04$) energy corrected milk (ECM) than GS. Cows BCS and BW were not affected by treatment. Treatment had no effect on milk yield, milk protein, lactose, and casein yield. Cows offered PC had lower ($P = 0.03$) milk fat yield (kg/d) compared with GS and tended to have a lower ($P = 0.05$) milk solids yield (milk fat + protein yield). Milk fat, protein, lactose, and casein concentration were not affected by treatment. Milk urea concentration was lower ($P = 0.01$) for cows offered PC than cows offered GS. There was a trend in higher SCC ($P = 0.06$) for the PC group compare to GS.

The effect of treatment on rumen fermentation parameters is shown in Table 3. Cows offered PC had lower ($P < 0.01$) rumen NH_3-N than GS, while rumen pH was not affected by treatment. Total VFA tended to be lower ($P = 0.07$) in cows fed PC compared to cows fed GS. The acetic: propionic acid ratio and the concentrations of acetic, propionic, butyric and iso-butyric acid concentrations were not affected by treatment. Valeric and iso-valeric acid concentrations were lower ($P < 0.01$) in cows fed PC. Partially replacing grass silage with biorefined press cake silage did not affect any of the blood metabolites analysed (glucose, NEFA and BHB; Table 4).

3.3. N and P partitioning study

The effects of partial replacement of grass silage with biorefined press cake silage on N and P partitioning are shown in Table 5. Feed N

Table 3
The effect of treatment on *in vivo* rumen fermentation parameters.

Item	Treatment		SEM	*P*-value
	GS [1]	PC [2]		
Rumen pH	6.83	6.84	0.024	0.70
NH_3-N (mmol/L) [3]	4.18	3.48	0.167	<0.01
VFA (mmol/L)				
Total VFA	120.66	115.39	2.097	0.07
Acetic: propionic acid ratio	3.04	3.02	0.023	0.54
Acetic acid	75.00	72.12	1.343	0.12
Propionic acid	24.50	23.75	0.393	0.18
Butyric acid	15.15	14.52	0.361	0.22
Iso-butyric acid	1.06	0.96	0.039	0.11
Valeric acid	2.90	1.78	0.062	<0.01
Iso-valeric acid	2.86	2.15	0.077	<0.01

[1]Grass silage treatment (14 kg DM of grass silage + 7.2 kg DM standard concentrate +0.44 kg DM soyabean meal).
[2]Press cake treatment (5 kg DM grass silage + 9 kg DM press cake silage + 7.2 kg DM standard concentrate + 0.44 kg DM soyabean meal).
[3]Rumen ammonia nitrogen.

Table 4
The effect of treatment on blood metabolites.

Item	Treatment		SEM	*P*-value
	GS [1]	PC [2]		
Glucose (mmol/L)	3.25	3.14	0.054	0.19
BHB (mmol/L)[3]	1.02	0.92	0.054	0.21
NEFA (mmol/L)[4]	0.11	0.12	0.003	0.65

[1]Grass silage treatment (14 kg DM of grass silage + 7.2 kg DM standard concentrate +0.44 kg DM soyabean meal).
[2]Press cake treatment (5 kg DM grass silage + 9 kg DM press cake silage + 7.2 kg DM standard concentrate + 0.44 kg DM soyabean meal).
[3]Beta-Hydroxybutyrate (BHB).
[4]Non-Esterified Fatty Acid (NEFA).

intake was lower ($P < 0.01$) for cows fed PC than for those offered GS. Fecal and urinary N excretion (kg/d) were lower ($P < 0.01$) when PC was offered compared to GS. No effect of treatment was observed on milk N excretion. The proportion of N excreted into milk was higher ($P < 0.01$) for cows offered PC than for those offered GS. Treatment had no effect on the proportion of N excreted into milk and urine. Total N excreted as a percentage of N intake was lower ($P < 0.01$) for cows fed PC while cows fed PC had a higher ($P < 0.01$) NUE compared to cows fed GS.

Cows fed PC had a lower ($P < 0.01$) dietary P intake had a lower ($P < 0.01$) fecal P output (kg/d) than cows fed GS. Urine and milk P output (kg/d) did not differ significantly between treatments. The proportion of P excreted in the feces was lower ($P < 0.01$) for cows offered PC than for those offered GS. However, the partial replacement of grass silage with biorefined press cake did not affect the proportion of P excreted in urine or milk and the phosphorus utilization efficiency. Total P excretion as a percentage of P intake was lower ($P < 0.01$) for PC compared to GS.

3.4. *In vitro experiment: DM disappearance, fermentation parameters, gas and methane production*

The effect of treatment on DM disappearance, vessel liquid pH, NH_3, VFAs concentrations, gas and CH_4 output are presented in Table 6. Dry matter disappearance of silage components and total diet was less ($P = 0.01$; $P = 0.02$) for PC than GS. There was no difference between treatments for vessel liquid pH. However, daily NH_3 concentration was altered by treatment; NH_3 was lower ($P < 0.01$) in PC compared to GS. No significant difference was found between treatments in total VFA, acetic, propionic, butyric, iso-butyric, valeric acid production, and in the acetic: propionic acid ratio. Iso-valeric acid production tended to be

Table 5
The effect of treatments on nitrogen and phosphorus partitioning.

Item	Treatment		SEM	*P*-value
	GS [1]	PC [2]		
Intake (kg/d)				
Feed N	0.71	0.61	0.036	<0.01
N output (kg/d)				
Milk	0.19	0.18	0.013	0.50
Feces	0.23	0.19	0.020	<0.01
Urine	0.27	0.22	0.015	<0.01
N partitioning[3]				
Milk	0.27	0.32	0.006	<0.01
Feces	0.35	0.34	0.011	0.40
Urine	0.37	0.34	0.013	0.10
N excreted (%) [5]	72.66	68.09	0.611	<0.01
NUE (%) [7]	27.33	31.90	0.611	<0.01
Intake (kg/d)				
Feed P	0.095	0.089	0.0001	<0.01
P output (kg/d)				
Milk	0.026	0.025	0.0007	0.28
Feces	0.066	0.052	0.0026	<0.01
Urine	0.0039	0.0034	0.00005	0.18
P partitioning [4]				
Feces	0.68	0.57	0.017	<0.01
Milk	0.26	0.25	0.0007	0.28
Urine	0.030	0.027	0.0052	0.42
P excreted (%) [6]	72.75	61.77	2.608	<0.01
PUE (%) [8]	28.03	28.83	0.737	0.45

[1]Grass silage treatment (14 kg DM of grass silage + 7.2 kg DM standard concentrate +0.44 kg DM soyabean meal).
[2]Press cake treatment (5 kg DM grass silage + 9 kg DM press cake silage + 7.2 kg DM standard concentrate + 0.44 kg DM soyabean meal).
[3]N partitioning = N out [faces, urine, milk (kg/d)]/N intake (kg/d).
[4]P partitioning = P out [faces, urine, milk (kg/d)]/P intake (kg/d).
[5]N excreted = [faeces + urine output (kg/d)]/N intake (kg/d) * 100.
[6]P excreted = [faeces + urine output (kg/d)]/P intake (kg/d) * 100.
[7]NUE, nitrogen utilization efficiency = [milk output (kg/d) /N intake (kg/d) * 100].
[8]PUE, phosphorus utilization efficiency = [milk output (kg/d) / P intake (kg/d) * 100].

Table 6
The effect of treatments on apparent digestibility, rumen fermentation, total gas and methane output using *in vitro* rumen simulation technique (RUSITEC).

Item	Treatments		SEM	*P*-value
	GS[1]	PC[2]		
DM disappearance % (s)	84.40	66.95	3.090	0.01
DM disappearance % (t)	77.92	65.45	2.402	0.02
Fermentation parameters				
pH	6.83	6.92	0.041	0.18
NH_3 (mmol/L)	2.19	1.09	0.153	< 0.01
VFA production (mmol/L)				
Total VFA	78.27	78.10	3.351	0.97
Acetic: propionic acid ratio	1.95	2.00	0.074	0.63
Acetic acid	42.11	45.09	1.820	0.31
Propionic acid	23.42	24.04	1.025	0.67
Butyric acid	8.34	7.64	0.641	0.48
Iso-butyric acid	0.90	0.94	0.092	0.76
Valeric acid	2.11	1.93	0.071	0.10
Iso-valeric acid	2.43	2.16	0.096	0.05
Total gas production (L/d)	1.31	1.26	0.272	0.90
Methane (mmol/d)	6.61	5.71	1.248	0.63

[1]S = silage; t = silage + concentrate + soyabean meal.
[2]Grass silage treatment (10 kg DM of grass silage + 5.14 g DM standard concentrate + 0.3 g DM soyabean meal).
[3]Press cake treatment (3.4 g DM grass silage + 6.6 g DM press cake silage + 5.14 g DM standard concentrate + 0.3 g DM soyabean meal).

lower ($P = 0.05$) in PC compared to GS. Total gas and CH_4 production was not affected by treatment.

4. Discussion

The objective of this study was to determine the effect of partial replacement of grass silage with biorefined press cake silage on DMI, milk production and composition, rumen fermentation, N and P excretion of early lactation Holstein Friesian dairy cows and on *in vitro* digestibility, rumen fermentation, total gas and methane output. In this study, press cake silage was used as a partial replacement for grass silage and dietary treatments were not isonitrogenous. Diets offered did however meet the nutritional requirements of the cows used in the study.

In temperate regions of the world, milk is produced primarily from grassland which allows farmers to capitalize on this low-cost feed resource (O'Brien et al., 2018). Although this represents a competitive advantage over other countries' production systems, it also creates its own set of challenges from an environmental perspective. These challenges include high N losses due to low NUE and resource use efficiency, as a result of low efficiency in grass utilization at farm level (approximately 60% in Ireland; Ryan et al., 2011; Läpple et al., 2012; Kelly et al., 2020). The biorefinery process represents an opportunity to meet those challenges by producing a range of products from fresh grass, thereby improving its utilisation (Franco et al., 2019). Press cake and press juice are the main products obtained through this process. The press juice can be further processed recovering different by-products, such as green protein concentrate, grass whey, FOS, and de-FOS whey (Ravindran et al., 2021, 2022). The press cake obtained from this process can have multiple applications, such as solid fuel or substrate for biogas production (Xiu and Shahbazi, 2015; Ravindran et al., 2022), but higher added value can be obtained by feeding it to dairy cows (Damborg et al., 2019), with reductions in N and P excretion possible (Pijlman et al., 2018).

4.1. In vivo experiment

4.1.1. DMI, feed efficiency, BCS, BW, milk production, milk composition, rumen fermentation and blood metabolites

In the biorefinery process, grass or silage is mechanically separated into a liquid fraction and a solid fraction (press cake). Soluble components, such as minerals and proteins, are concentrated in the liquid, leaving the solid fraction higher in DM and NDF and lower in CP compared to unrefined silage (McEniry and O'Kiely, 2013; Franco et al., 2019; Savonen et al., 2020). In this study, the DMI of the PC diet was lower than that of the GS diet, likely due to its higher DM and NDF content. This has widely been reported to have a physical effect on rumen fill and the rate of digestion, in turn, reducing voluntary feed intake (Allen, 2000). In the current study, it was hypothesized that partially replacing grass silage with press cake silage would not affect milk production and composition due to a more efficient use of the insoluble nutrients remaining in the silage after the biorefinery process. The partial replacement of grass silage with press cake silage caused a difference in nutrient supply, in particular, the PC diet had a higher NDF and lower CP content compared to the GS diet. This may not only be due to the biorefinery process, but also to the difference in harvesting time for the grass silage compared to the press cake silage. However, these differences were not significant enough to alter milk production and milk quality, as cows that were offered PC maintained the same milk yield and milk component concentrations as cows offered GS. With the increase in NDF concentration of the PC diet, milk fat yield (kg/d) was expected to increase as a consequence of increased production of acetic acid in the rumen (McDonald et al., 2011). However, in the present study, treatment did not affect acetic acid concentration in the rumen and cows fed PC had a lower milk fat production compared to cows fed GS. The ECM was lower for PC compared to GS, indicating a reduction in milk production potential of the PC compared to GS. Similar results were reported by Savonen et al. (2020) where lactating dairy cows were offered two levels of refined silage (pulp; 25% of the total forage and 50% of the total forage).

Milk urea concentration is highly correlated with the protein content of the diet and the decrease of CP concentration in PC led to a lower milk urea concentration, in agreement with the findings of other studies (Frank and Swensson, 2002; Whelan et al., 2012; Savonen et al., 2020).

Feeding PC had no effect on rumen pH and VFA production. Rumen NH_3-N concentration was lower in cows offered PC, due to the lower CP in the silage after the biorefinery process. Savonen et al. (2020) observed a reduction in protein balance in the rumen of cows fed press cake silage. Therefore, feeding press cake silage to dairy cows may have increased the concentration of RUP and can help explain the lower concentration of NH_3-N in the rumen fluid observed in the current study (McDonald et al., 2011).

4.1.2. N and P partitioning study

The loss of N from livestock production system represents a major challenge for the sector (Environmental Protection Agency EPA, 2020). In the current study, changes in N excretion and N partitioning were observed when grass silage was partially replaced with press cake silage, although N excreted in milk and the proportion of N excreted in urine were not affected. The lower N concentration in the PC diet and the lower DMI in the PC group reduced the N intake compared to the GS group. Several studies have reported a positive linear relationship between N intake and N excreted in feces and urine (Castillo et al., 2001; Mulligan et al., 2004; Carmona-Flores et al., 2020). The lower N intake of the PC group led to a lower fecal and urinary N excretion with potential environmental benefits, as less N is potentially lost to the environment. The CP concentration of the diet is highly related to NUE and the decrease in CP concentration may be considered beneficial in increasing NUE (Huhtanen et al., 2008). Powell and Rotz (2015) reported that for every percent decrease in dietary CP, NUE increased by approximately 2 per cent, as more N was excreted into milk, and less N excreted in the manure. In the present study, cows fed the lower CP PC diet (15%) compared to the GS diet (18% CP) had an NUE of 31.9%, compared to the GS diet at 27.3%. There was also a higher percentage of N excreted in milk of cows offered PC (32% in PC compared to 27% in GS). Similar results were observed by Savonen et al. (2020), when 50% of the silage in the diet was replaced with press cake silage. The authors also found a linear increase in NUE with increasing levels of press cake silage in the diet.

Phosphorus is an essential mineral for animal production and reproduction (Wang et al., 2014). The utilization of P in dairy cows is inefficient, with less than 40% of dietary P intake used by the animal, with the rest being excreted, primarily in feces, leading to concerns about the pollution of surface water and rapid growth of algae populations that can compromise the surviving and productivity of fish and other animal life (Knowlton and Herbein, 2002). In the present study, dietary P intake was lower in PC compared to GS due to the lower P content of the diet and the lower overall intake. As a consequence of a lower P intake (Reid et al., 2015), cows fed PC had a lower P output in feces and lower total P excretion as a percentage of intake compared to cows fed GS in agreement with the research of Pijlman et al. (2018).

4.2. In vitro experiment

The effect of partial replacement of grass silage with press cake silage on *in vitro* rumen pH and NH_3 concentration is consistent with the *in vivo* rumen fermentation parameters. The NH_3 concentration in the fermented liquid was lower in the vessels fed PC compared to vessels fed GS. With the loss of the soluble protein during the biorefinery process, the remaining CP in the press cake silage is expected to be bound to the cell walls and therefore is less digestible, resulting in a lower production of NH_3 (Xiu and Shahbazi, 2015; Damborg et al., 2018). Similar findings were reported by Sarnataro et al. (2019) using the RUSITEC system to

compare a biorefinery by-product from alfalfa silage, the original alfalfa silage and hay. Apparent total tract digestibility was greater for PC compared to GS in the current *in vivo* study. However, in the RUSITEC study, DM disappearance of PC silage fraction and the total diet was lower compared to GS. This contradiction may be due to the differences arising between the *in vitro* technique and *in vivo* studies as highlighted by by Czerkawski and Breckenridge (1977). The lack of absorption, the difference in fluid and particle passage rate and the lack of post ruminal digestion are some of the many differences that exist between the RUSITEC system and *in vivo* (Meyer et al., 1971; Hristov et al., 2012).

5. Conclusions

Cows offered PC maintained similar milk production and composition compared to cows offered GS despite higher NDF, lower CP concentration and lower DMI. Furthermore, cows receiving the PC diet had a higher NUE and lower N and P excretion as a percentage of intake. Partially replacing grass silage with press cake silage did not affect *in vitro* total gas and CH_4 output, however the NH_3 concentration in the fermented liquid was reduced. This study suggests that press cake silage can partially replace grass silage in the diet of dairy cows with potential beneficial effects on the environment and without compromising animal productivity. The differences in origin and harvesting times of that silages are recognised as limitations of the study.

CRediT authorship contribution statement

E. Serra: Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Data curation. **M.B. Lynch:** Conceptualization, Resources, Supervision, Writing – review & editing, Funding acquisition. **J. Gaffey:** Conceptualization, Project administration, Funding acquisition, Writing – review & editing. **J.P.M. Sanders:** Conceptualization, Funding acquisition, Writing – review & editing. **S. Koopmans:** Conceptualization, Writing – review & editing. **M. Markiewicz-Keszycka:** Data curation, Writing – review & editing. **M.H. Bock:** Investigation, Data curation, Writing – review & editing. **Z.C. McKay:** Writing – review & editing. **K.M. Pierce:** Conceptualization, Resources, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare to have not stated any conflicts of interest, financial or otherwise.

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Production of Green Biorefinery Protein Concentrate Derived from Perennial Ryegrass as an Alternative Feed for Pigs

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Abstract: Perennial rye grass is a widely used forage species in Ireland, on which the ruminant sector of agriculture is heavily dependent. While this species of grass is the primary source of fodder for cows, it is also abundant in plant protein, which could form a potential alternative ingredient in monogastric animal feed using a green biorefinery approach. In this study, perennial rye grass was processed using a novel biorefining process to extract value added products including protein as a potential replacement for soybean meal in monogastric feeds. Feed trials were conducted on a commercial farm with 55 weaner pigs for 31 days until slaughter. The diets comprised a control and a trial diet which integrated the green biorefinery protein concentrate. The effects of the new diet were determined by measuring the daily feed intake (DFI), average weight gain (AWG) and feed conversion ratio (FCR). Amino acid profiles of grass protein concentrate and soybean meal were comparable, with the latter having a slightly higher amount of total protein content, lysine and cysteine. The DFI and ADW indicated that the treatment diet was superior to the control. DFI for the treatment diet (1.512 kg/d) was 8% higher than the control diet (1.400 kg/d) by the end of the trial. Additionally, the ADW for the treatment diet was 6.44% higher than that achieved in the control sample. Meanwhile, FCR calculations indicated that the treatment diet is just as efficient as the conventional diet. Overall, the results of the study indicate positive potential for perennial ryegrass-derived green biorefinery protein concentrate as an alternative protein source for pig feed formulations in Ireland.

Keywords: green biorefinery; perennial rye grass; grass protein concentrate; monogastric feed; protein recovery

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1. Introduction

The continuing exponential increase in population coupled with a growing consumer demand for edible protein has resulted in a significant intensification of agriculture over recent decades, raising questions about the sustainability of the livestock sector. To meet this growing demand, the meat industry in Europe has become heavily dependent on the importation of protein-rich plant-based feed additives such as maize and soybean [1]. Ireland has a vibrant livestock industry comprising cattle, dairy, pig and poultry sectors [2]. Sustaining this sector is particularly reliant on imports of animal feed and related additives. In 2018, Ireland imported approximately 5.1 million tonnes of animal feed materials. Almost two-thirds of the animal compound feeds in Ireland are imported, compared to the UK (37%), France (27%) and Germany (26%). The pig, poultry and dairy sectors are particularly dependent on the import of genetically modified (GM) soy, maize and their by-products forming essential ingredients in animal feed formulations [3]. Almost 2.7 m tonnes of soya and maize GM products were imported into Ireland for animal feed applications in 2018, constituting approximately 50% of total feed imports. Up to 90% of the soybean and 80% of maize products are imported from Argentina, Brazil, Canada

and the USA [4]. Significant quantities of non-GM maize and oilseed rape meal are also imported from continental Europe, including Ukraine [5].

According to new regulations by the EU, 30% of the protein for monogastric feed purposes must be sourced locally [6]. Although soy can be grown in Europe, it is usually only cultivated for commercial exploitation in the southern and eastern regions. Furthermore, soy cultivation in these regions cannot meet the animal feed demand of the whole of Europe. Therefore, northern Europe is heavily reliant on soybean meal imports [3]. Sources such as seaweed, mussels, insects and forage crops have been extensively investigated as potential sources of protein to decrease the dependence on soy imports for monogastric feed applications [7]. The European Commission published a report in 2018 detailing the development of plant proteins in the EU. Accordingly, this report investigated the supply and demand of protein within the EU. In 2016/17, the EU demand for plant protein amounted to 27 million tonnes of crude protein. Five major sources of plant protein were identified in this report, i.e., pulses, soybean complex, rapeseed complex, sunflower complex and dried fodder legume. Out of all these protein sources, soybean complex (soybeans, soybean oil and soybean meal) contributed to 14.3 million tonnes of the crude protein demand, with a huge share being imported. Meanwhile, a major share of the dried fodder legume, rapeseed complex and pulses were sourced from within the EU. The overdependence of the European Markets on imported soybean complex has called for member states to develop national protein strategies to guarantee long-term, sustainable food security.

EU member states such as Germany, France, Netherlands and Nordic countries such as Denmark and Finland have developed 'National Protein Strategies' with the aim to establish sustainable food security, efficient and resilient circular supply chains, support sustainable innovation and conserve natural resources and ecosystems. A common strategy among Germany, France and Finland is to encourage extensive cultivation and production of legumes as a potential source of protein [8,9]. This is in consensus with the EU report on sustainable sources of plant protein which explores the agronomic, environmental and climate benefits of legumes. Meanwhile, Denmark focuses on exploiting seaweed, non-native urchins and mussels as alternative sustainable sources of protein [10]. All the EU nations discussed in this section have emphasised the need for additional funding for protein crop strategies and R&D activities for developing alternative sustainable sources of protein for food and feed applications. Another common objective among these nations is the development of circular economies and sustainable supply chains to ensure that the products are sustainable and competitive in the global market.

Meanwhile, the EU strategy for the development of plant protein, known as the Supranational Protein Strategy, aims to support EU nations to grow protein crops and develop supply chains while creating a partnership between the government, industry and academia, encouraging customer behaviour towards sustainable food choices, developing practices and policies towards sustainable production and tapping into the benefits of sustainable production systems and agri-ecological practices. To achieve these aims, the EU has devised measures to support farmers in growing plant proteins by coordinating research into plant protein sources and providing funding to innovative projects that explore the sustainable production of plant protein, improving knowledge transfer, technical support and investments to provide rural development support on farms and promote the benefits of plant protein with respect to nutrition, health, climate and environment [11].

Circular bioeconomy relies on sustainably produced biomass as raw materials for the co-production of various products including food, feed, materials, chemicals and biofuels. Proteins for food and feed application are of great interest to establish a viable bioeconomy model which has the potential to be scaled-up to commercial operation. Agro residues are particularly interesting from a biorefining perspective since most oil seeds such as sunflower and rapeseed, which are widely cultivated in Europe for their oil content, leave behind protein-rich press cake on processing [12,13].

Wheat is the most cultivated crop in the EU and is rich in protein content [14]. This is followed by maize, barley, sunflower, rapeseed, soybean, pea, millet etc. [15]. Protein sources have been classified into five groups based on their protein content. Group 1 contains more than 50% of protein dry weight. A few examples of these protein sources are soybean protein and rapeseed protein concentrates. Protein sources which contain 25–45% of protein in their dry weight are classified as group 2 (e.g., rapeseed press cake, soybean meal, sunflower seed meal, microalgae etc.). Group 3 encompasses protein sources which have a protein content ranging between 10–20% dry weight. Examples include rapeseed hull, soybean pods, beet leaves and fresh grass. Meanwhile, protein sources with protein content between 5–10% dry weight are categorised as group 4 (e.g., rape straw, soy straw, corn stover, etc.). Lastly, protein sources where protein contributes to less than 5% of its dry weight is falls under group 5. An example of such a protein source is wheat straw [16].

Nutritional value and digestibility are two major factors that influence the utilisation of a protein source for food and feed applications. Plant-based proteins do not necessarily provide nutritional value in balanced proportions, which is a prerequisite for animal feed formulations. Essential amino acids such as lysine, cysteine and methionine have previously been found to be lacking in plant-based proteins [17]. The Protein Digestibility Corrected Amino Acid Profile Score (PDCAA) is a metric used to measure the nutritional value of protein derived from various sources. In the current scenario, soybean meal and canola have been identified as the best sources of plant protein based on their PDCAA scores. For this reason and for reasons of low-cost availability, soybean meal has been the major source of protein exploited for feed applications, followed by maize [18]. On the other hand, plant protein digestibility is low, which is a deterrent in their use in food and feed application. Furthermore, processing operations can further affect digestibility due to the loss of moisture content and the formation of complex disulphide bonds within the protein.

Soybean meal (SBM) is the primary plant-protein source for swine diets. The amino acid (AA) profile of SBM is well-balanced and complements the AA profile of grains such as corn and wheat, and these AAs are highly digestible for pigs [3]. The energy content of SBM has been reported as 3619 kcal/kg digestible energy (DE) and 3294 kcal/kg metabolizable energy (ME), which suggests that SBM has 105% and 97% of corn grain DE and ME values, respectively [19]. SBM plays an important role in animal feed production and has become one of the primary crops cultivated by farming communities across the world. However, the popularity of this oil seed for its protein content has resulted in daunting environmental problems. Since soy plants only produce one yield in their lifetime, their cultivation requires more land to meet demand. In 2017, 123.6 million hectares of land were used to grow 352.6 million tonnes of soybeans [20]. In the tropical countries where soy is largely produced, demand for soybean has resulted in vast areas of virgin land being cleared to grow this crop. This has led to widespread deforestation in South America, especially in Brazil, Argentina, Bolivia and Paraguay. In 2018, 57 million hectares of forest land were dedicated to soy production. Worldwide, soy cultivation takes up an area the size of France, Belgium, Germany and the Netherlands combined [21].

Along with deforestation, other concerns related to global soy production include carbon emissions, soil erosion and strained water resources. Conversion of forest land into agricultural land is one of the greatest contributors to carbon emissions. Forests absorb and store huge amounts of carbon dioxide which is released into the atmosphere when they are cleared to grow crops like soy. Additional emissions are associated with mechanised processing and export-related food miles [22]. Meanwhile, soya-related soil erosion is caused by the intensive agricultural practices such as ploughing and intensive irrigation. The lack of tree cover makes the land susceptible to wind which results in loss of topsoil. Over the course of time, agricultural land wanes in fertility, leading to a decrease in productivity threatening long-term global food security [23].

The environmental problems raised by global soy production call for drastic innovative measures to meet the demand for protein sources for animal feed applications. Alternative

examples of other plant species that are viewed as potential plant protein sources include sunflower meal, beans, ground nuts, peas (pigeon peas, cow peas and chickpeas), sesame and green grains. However, from a national perspective, the climatic conditions in Ireland do not support the growth of soy and maize. To circumvent this problem, the EU and Ireland have partnered together through the Protein Aid Scheme launched in 2015 to subsidise farmers to grow other protein-rich crops such as beans, peas and lupins. However, only 8100 hectares in Ireland were dedicated to growing peas and beans in 2019. It is therefore safe to assume that this step, although promising, is unlikely to provide a viable solution to animal feed applications in the near future. On the other hand, grasslands constitute over 90% of the total agricultural lands in Ireland. Perennial rye grass, Italian rye grass and white clover are the three varieties of grass found in Ireland. Of these, perennial rye grass is the most commercially important form of grass as the other grass varieties are not commonly used.

Perennial rye grass is a temperate grass that is commonly found across Europe. This type of grass is rich in minerals and well-maintained crops have enough metabolizable energy. The climatic conditions in Ireland support the growth of various varieties of grass. The Republic of Ireland had a total of 4.9 Mha of grasslands in 2016 [24]. The agricultural economy of Ireland is heavily reliant on grasslands, in particular perennial rye grass varieties, and plays an important role in the ruminant industry [25]. Currently, the commercial application of this type of grass is confined as a cattle feed [26]. With a high metabolisable energy of 12.2 MJ/kg DM on average, this species of grass is the primary source of fodder for cows [27]. However, grass is also abundant in value-added products such as protein and can therefore form a potential raw material for small-scale green biorefinery processes [28].

Kromus et al. (2003) [29] describes green biorefinery as a concept to utilize green (grassland) biomass as raw material for the production of biobased products such as proteins, lactic acid, fibre and energy (via biogas). Green biorefineries process protein-rich green leafy biomass including grass (e.g., ryegrass, clover and lucerne) and fresh leaves (e.g., potato or beet leaves). The Green Biorefinery concept is currently at an advanced stage of development in several European countries, especially Germany, Denmark, Switzerland, the Netherlands and Austria. In 2001, the first green biorefinery began operation in Switzerland with a processing capacity of 5000 tonnes dry matter of grass per year [30] (Xiu and Shahbazi, 2015). BioWert, Gramitherm and Newfoss are among the commercial partners currently implementing green biorefineries in Europe, with a focus on non-protein products such as technical fibres for composites, insulation materials and paper [31]. Focusing specifically on efforts which have been undertaken to use green biorefineries to improve the protein availability of grasses, work in Denmark through Aarhus University and Netherlands through Grassa BV is the most advanced. The main focus of this approach is to separate soluble protein into a separate fraction which could be used by monogastric animals or in future plant food applications, while leaving sufficient protein within the fibres to serve as a high-quality ruminant feed. For example, through the Danish OrganoFinery project, a fermentation technology using the addition of a specific lactic acid bacterium for precipitation of proteins in the juice was developed with the resulting protein paste containing 5–7% of lactic acid, which was reported to be beneficial for the gut health of poultry and pigs [32]. A more recent study by Aarhus University presented a process using a screw press to separate white clover, red clover, lucerne and perennial ryegrass into a press cake and green juice fraction, with precipitation of protein from the juice performed by either a two-step heat precipitation (a) or acidic precipitation (b) [33]. A recent Danish study focused on biorefinery-extracted protein from organic grass-clover as an input to pig feed diets, found that the meat percentage measured at slaughter increased linearly with inclusion of grass-clover protein in the feed [34]. Meanwhile, two recent studies, one from Denmark and one from the Netherlands, have investigated green biorefinery press cake as an alternative feed in ruminant diets, with both studies demonstrating a comparable performance compared with un-refined silage, with reduced levels of n and p in cattle

excrement [35,36]. These findings point to the potential of green biorefining as a route for increasing the protein potential of grassland.

The aim is of this study was to determine the potential perennial rye grass protein as a monogastric feed protein additive. The authors could not find any study that investigated the application of protein derived from perennial rye grass in feed applications. In addition, no such previous biorefining study has previously been undertaken in Ireland. Therefore, in this study, an innovative grass biorefining process was employed to extract green protein concentrate from perennial rye grass. The novel grass protein was analysed for its nutritional content and amino acid profile. Indicators such as daily feed intake, average daily weight gain and feed conversion efficiency were calculated. Grass protein was tested as a potential protein supplement in animal feed for pigs alongside conventional soybean meal to determine the performance of green protein feed in comparison with a conventional weaner diet.

2. Materials and Methods

2.1. Preparation of Grass Protein Concentrate

Fresh perennial rye grass was harvested from farms located in West Cork. The feedstock was processed using a novel green biorefinery process developed by Grassa BV. A schematic of the protein extraction process is provided in Figure 1. Specific details on the protein extraction process cannot be discussed in detail due to proprietary concerns. Briefly, fresh grass was loaded into the biorefinery via a loading dock washed with water upon entry to remove dirt, sand and impurities. The grass then underwent mechanical fractionation via an extruder which produced two primary products, a press cake and a green juice. The press cake, containing approximately 50–60% of the original, primarily insoluble protein, was ensiled and baled, serving as low emission feed for ruminants, with a high nitrogen use efficiency. The remaining 40–50% of protein was pressed into the green juice, along with nutrients, minerals and mainly fructan sugars. Heat coagulation via heat exchangers solidified the protein contained in the juice, which was then separated by vacuum filter. This green protein juice could be used directly as an input to wet feeding but in this trial was further dried to approx. 90%DM via belt dryer. This product, a green protein concentrate, can now be storable for more than one year and was integrated within pig feed rations for this study. The residual grass whey can be further processed to extract high value fructans and produce a mineral concentrate.

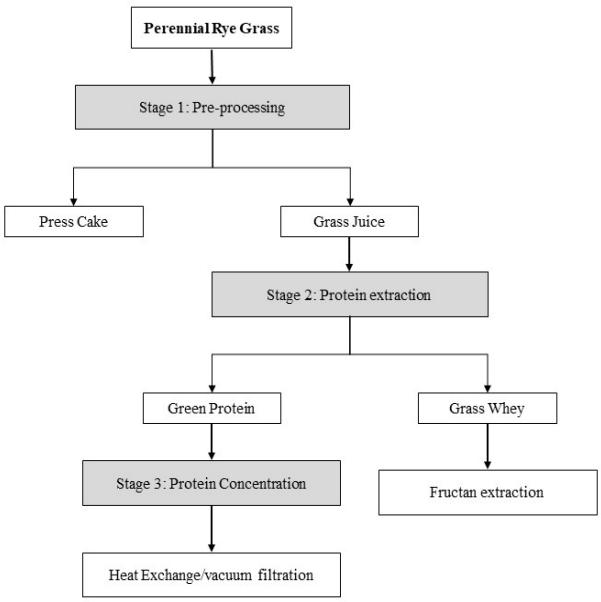

Figure 1. Schematic of protein extraction from perennial rye grass.

2.2. Characterisation of Perennial Rye Grass Protein Concentrate

Samples of green protein concentrate were subjected to proximate analysis. Proximate analysis was done by Dairy Gold analytical laboratory. Additionally, the amino acid profile of green protein concentrate was analysed at Sciantec, North Yorkshire, UK. The AA contents were measured by cation exchange chromatography after acid hydrolysis for 24 h [37].

2.3. Protein Concentrate Pig Feed Trails

To examine the feasibility of the grass protein as a potential replacement for soybean meal in pig feed diets, dry feed trials on a commercial pig farm were conducted to compare pig performance between control groups and treatment groups. Prior to the trial, the green protein concentrate was subjected to compositional analysis to assess the nutritional value. This information was used to replace conventional ingredients of the feed such as soybean meal and barley. The treatment feed was prepared to meet the nutritional requirements of the weaner pigs, details of which are provided in Table 1. The weaner diet was formulated on standardised ileal digestible amino acids. Protein is provided in the diet to supply amino acids (the building blocks of protein). There are 20 primary amino acids. Half of these are essential, in that they cannot be synthesised by the pig. The others are termed non-essential, as they can be synthesised in the body.

Table 1. Diet formulation for weaner pigs.

Nutrients	Concentration
Protein (%)	18.63
Oil (%)	5.77
Fibre (%)	3.11
Ash (%)	6.08
DE (MJ/kg)	14.42
NE (MJ/kg)	10.25
Lysine (%)	1.27
ILD Lysine (%)	1.15
Methionine (%)	0.42
Calcium (%)	0.75
Dig Phosphorus (%)	0.35
Sodium (%)	0.24
Vitamin A (IU/kg)	12,000
Vitamin D3 (IU/kg)	2000
Vitamin E (IU/kg)	125
Biotin (mcg/kg)	150
Phytase (FTU/kg)	1000

The dry feed trial was a comprehensive feed trial which focused on late stage finishing weaner pigs entering second stage weaner accommodation, aged nine weeks old and weighing 20 kg on average. The pigs were split into a treatment group and a control group with 54 pigs and 55 pigs, respectively, for approximately 30 days until slaughter. The control feed consisted of wheat, maize, barley, molasses, SBM, soy oil, soy hull and minerals in recommended amounts. The treatment feed, on the other hand, comprised green protein concentrate replacing a proportion of soybean meal, barley, and wheat by 27.3%, 25% and 8%, respectively, in comparison to the control. Additionally, the fraction of soy hull was increased by 33.3% in the treatment compared to the control. The compositions of the treatment feed and control feed are provided in Table 2. Weekly weigh-ins and feed intakes were recorded to allow the calculation of daily feed intake, average daily gain and feed conversion ratio for each treatment.

Table 2. Composition of control and treatment feed.

Raw Material	Control	Treatment
Barley (%)	30.00	22.50
Maize (%)	10.00	10.00
Wheat (%)	25.00	23.00
Molasses (%)	2.00	2.00
Hipro Soya (%)	22.00	16.00
Grass Protein Pellets (%)	-	15.00
Soya Hulls (%)	1.00	1.50
Lactoflo (%)	2.50	2.50
Soya Oil (%)	3.70	3.70
100 Weaner + Vita GP (3.8%)	3.80	3.80

2.4. Daily Feed Intake (DFI), Average Weight Gain and Feed Conversion Ratio

The daily feed intake for each treatment was calculated as follows:

$$\text{Daily Feed Intake} = \frac{\text{Total Feed Intake}}{\text{Number of pigs on treatment}}$$

Total feed intake is the feed delivered less the feed remaining at the end of the trial [38].

Meanwhile, weigh-ins of the pigs were conducted during the whole period of the trial, at the start and at the end of every week until the end of the trial, in order to calculate average daily weight gain.

Feed conversion ratio was calculated as follows [39]:

$$\text{Feed Conversion Ratio} = \frac{\text{Daily Feed Intake}}{\text{Average Daily Weight Gain}}$$

3. Results

3.1. Characterisation of Green Protein Concentrate

Proximate analysis was performed to determine the different components in the green protein concentrate such as crude fibre, ash, protein, starch and total solids. Accordingly, green protein concentrate comprised a crude fibre content of 6.1%. Furthermore, the protein content was recorded to be 33.9%, ash content at 11.8% and oil within the range of 10.5 to 13.2%. Interestingly, no starch content was found in the protein concentrate. The finished feed appeared dark green in colour.

3.2. Amino Acid Profiling of Green Protein

Table 3 provides a comparison of soybean meal nutritional qualities with other oil seed meals and green protein concentrate. The complete amino acid profile of the green protein concentrate is provided in Table 4. Accordingly, the novel protein concentrate was rich in glutamate+ glutamin (3.6%) aspartate (3.1%), leucine (2.8%) and alanine (2.1%). Interestingly, the gross energy was found to be 4347 kcal kg^{-1}.

Table 3. Nutrient requirements for pigs at different stages of life [12].

Essential Nutrients	Creep 6–10 kg	Weaner 10–20 kg	Grower 20–50 kg	Finisher 50–100 kg	Dry Sow	Lactating Sow
Crude protein	23–25	19–22	18–20	16–17	13.5–13.8	17–18
Crude fibre	1–3	2–4	2–5	3–4	4–5	2–5
Lysine	1.3–1.5	1.25–1.35	1.0–1.1	0.85–0.95	0.6–0.7	1.0–1.15
Arginine	0.52–0.6	0.5	0.4–0.44	0.34–0.38	0.24–0.28	0.4–0.46
Histidine	0.46–0.53	0.44–0.47	0.35–0.39	0.30–0.33	0.21–0.25	0.35–0.40
Isoleucine	0.78–0.9	0.75–0.81	0.6–0.66	0.51–0.57	0.36–0.42	0.6–0.69
Leucine	1.43–1.65	1.38–1.5	1.1–1.2	0.94–1.01	0.66–0.77	1.1–1.27
Methionine + cystine	0.78–0.9	0.75–0.81	0.6–0.66	0.51–0.57	0.36–0.42	0.6–0.69

Table 3. *Cont.*

Essential Nutrients	Creep 6–10 kg	Weaner 10–20 kg	Grower 20–50 kg	Finisher 50–100 kg	Dry Sow	Lactating Sow
Phenylalanine + tyrosine	1.24–1.43	1.2–1.3	0.95–1.04	0.81–0.90	0.57–0.67	0.95–1.09
Threonine	0.78–0.9	0.75–0.81	0.6–0.66	0.51–0.57	0.36–0.42	0.6–0.69
Tryptophan	0.23–0.27	0.23–0.33	0.18–0.20	0.15–0.17	0.11–0.13	0.18–0.21
Valine	0.98–1.1	0.94	0.75–0.83	0.64–0.71	0.45–0.53	0.75–0.86

Figures are represented as g per 100 g of feed.

Table 4. Amino acid profile of green protein in comparison with perennial rye grass protein [40]. Reproduced from [40], Journal of Dairy Sciences: 2013.

Amino Acid	Green Protein Concentrate	Perennial Rye Grass
Alanine	2.12	7.8
Arginine	1.84	6.0
Aspartic	3.09	10.4
Cystine	0.18	1.4
Glutamic	3.58	12.3
Glycine	1.79	6.2
Histidine	0.65	2.2
Iso-leucine	1.48	4.7
Leucine	2.75	9.4
Lysine	1.81	4.6
Methionine	0.65	2.2
Phenylalanine	1.84	5.7
Proline	1.52	6.0
Serine	1.38	5.0
Threonine	1.50	5.2
Tryptophan	0.61	-
Tyrosine	0.99	3.4
Valine	1.87	6.5

3.3. Daily Feed Intake

During the trial, the daily feed intake was recorded at the end of every week. The treatment feed was very well received by the pigs, and they ate well. The average weight of the pigs at the start of the trials was recorded to be 1.079 kg for the control diet and 1.132 kg for the treatment diet. There was a steady increase in the feed intake for both control and treatment diets as expected. During the first week, the feed intake for the control feed was recorded to be 0.991 kg/d. In comparison, this figure was 1.022 kg/d for the treatment diet. As the trial progressed, the difference between the daily feed intake for the control diet and the treatment diet increased considerably. By the end of the trial, the daily feed intake for the treatment diet (1.512 kg/d) was 8% higher than the control diet (1.400 kg/d). Dung consistency was normal, as can be observed in Figure 2, where the feaces of the treatment pigs appeared green. Pigs were clean and no major differences were observed when compared to the pigs on control diets.

(**A**) (**B**)

Figure 2. Dung consistency of treatment (**A**) and control weaners (**B**).

3.4. Average Daily Weight Gain

During the trial, the weaner pigs were weighed individually at the start of the trial and at the end of the week thereafter. The superiority of the treatment diet over the control can be observed from ADG comparisons. On the control diet, the pigs gained 0.592 kg/day during the first week. This rate increased as the trial progressed with an average daily gain of 0.646 kg/day after the second week, 0.699 kg/day after the third week and 0.682 kg/day at the end of the trial. On the other hand, the average weight gain of pigs on the treatment diet started slowly at 0.577 kg/day by the end of the first week but increased substantially as the trials progressed. The average weight gain by the end of the second week was 0.683 kg/day, which increased to 0.729 kg/day. By the end of the trial, a high average weight gain of 0.742 kg/day was achieved.

3.5. Feed Conversion Ratio

The feed conversion ratio for the trial is provided in Table 5. The initial value for FCR in the first week was reported to be 1.67 for the control diet. However, this value was higher for the treatment diet (1.77). Nonetheless, the FCR values for the succeeding weeks for both treatment and control were found to be similar. In general, the feed conversion ratio for both control and treatment diets increased each week as the trial progressed. There was no comparable difference between FCR values of the control diet and treatment diet by the end of days 15, 21 and 31.

Table 5. Daily feed intake, feed conversion ratio and average daily gain of weaners on treatment and control diets.

Date of Weighing	Daily Feed Intake (kg/d)		Feed Conversion Ratio		Average Daily Gain (kg/day)	
	Treatment	Control	Treatment	Control	Treatment	Control
Period 1	1.022	0.991	1.77	1.67	0.577	0.592
Period 2	1.247	1.182	1.83	1.83	0.683	0.646
Period 3	1.386	1.301	1.90	1.86	0.729	0.699
Period 4	1.512	1.400	2.04	2.05	0.742	0.682

4. Discussion

4.1. Green Protein Concentrate as an Additive for Pig Feed Preparation

Perennial rye grass was subjected to biorefining to extract green protein for monogastric feed applications. The biorefining activities involved extrusion followed by protein extraction and coagulation using heat. All the processes involved in the preparation of the protein concentrate used environmentally friendly, sustainable strategies. Further details about the biorefining process cannot be discussed due to proprietary issues. The pig's gut is an important factor for health and consistent performance. 'Gut health' is an underestimated factor that not only acts as a digestive organ but also has an immunological function. Modern livestock animals are typically fed high concentrate nutrient-dense diets to meet nutrient requirements [41]. However, high-concentrated diets cannot meet physiological requirements, as animals need dietary fibre for optimal health and digestion. A minimal level of fibre is a prerequisite for optimal nutrition and ingredients metabolism. Crude fibre is a measure of the fermentable components of the feed. Although low in energy this indigestible carbohydrate is important for the gut health of pigs and poultry [42]. It was therefore important to assess the amount of crude fibre in the green protein. Determination of these components allows us to make legitimate comparisons of feeds based on nutritional composition. The crude fibre content in the green protein concentrate was similar to that found in soybean meal (Table 5).

The ash content in the green protein concentrate was found to be 11.8%. In comparison, reported values of ash content in soybean meal fall within the range of 4.5–6.4% [42]. A high ash content in green protein concentrates may be attributed to the presence of sand due to

improper washing of the grass feedstock. Ash content does not affect the digestibility of protein. On the other hand, high ash content can contribute to higher utilisation of digested protein [43].

Table 4 provides a comparison between the crude protein content of soybean meal and green protein concentrate. Soybean meal has a higher crude protein content which falls in the range of 44–48 g per 100 g. However, the crude protein in green protein concentrate was found to be 33.9%. This is an indication that soybean meal cannot be completely replaced by perennial rye grass protein concentrate when preparing pig feed rations. Another important aspect that has a significant impact on monogastric feed formulations is the synthetic amino acid content. There has been a steady increase in the use of synthetic amino acid in animal nutrition. More than half of the total global amino acid production is represented by animal feeds. Amino acids that are critical in the normal growth of the animal include lysine, threonine, tryptophan, methionine (and cystine), isoleucine, leucine, histidine, valine, arginine and phenylalanine (and tyrosine). However, the amino acids of greatest practical importance in diet formulations (i.e., those most likely to be at highest deficient levels) are lysine, tryptophan, threonine and methionine [44]. Table 1 represents the amino acid requirements and their respective levels in pig feed with respect to their age. Currently, soybean meal is considered as the best protein source for feed requirements for monogastric animals. Additionally, soybean meal provides a good amino acid balance due to the presence of high amounts of lysine, tryptophan, threonine and isoleucine. Furthermore, the digestibility of amino acids in soybean meal is high. While the total protein, lysine and cysteine contents are slightly higher in soybean meal in comparison with green protein concentrate, the methionine content and threonine content are at par. Besides, the latter has a higher percentage of crude fibre which, as mentioned earlier, is crucial for the pigs' gut health. From Table 5, the content of essential amino acids in the green protein concentrate is comparable with that of soybean meal.

From Table 4, it is evident that green protein concentrate contains significantly lower amounts of lysine and cysteine in comparison with soybean meal. It was therefore important to include soybean meal and soya hulls as part of the treatment diet to mitigate the deficient lysine and cysteine content in green protein. However, the methionine and threonine content in the green protein concentrate are comparable with that of soybean meal. Furthermore, the essential amino acid concentration (lysine, methionine, threonine and cysteine) in green protein concentrate was higher than that of sunflower meal, rapeseed meal and cottonseed meal, which are all sources of protein widely exploited as animal feed additives [45,46].

4.2. Daily Feed Intake

According to a study conducted by Pierozan, Agostini [47], the daily dry matter intake is dependent on the number of pigs per pen, type of feeder, origin and sex of the pigs. Pigs are generally fed in two modes: dry feed and wet feed. Both feeding modes have been connected with respective advantages. Approximately 70% of pigs in Ireland are on wet feed. Many producers use wet feeding as their units were built at a time when significant volumes of liquid by-products (e.g., liquid whey and skim milk) were readily available. Wet feeding offered the potential to feed such by-products with a balancer, thereby providing a cheap balanced diet. A study performed by Zoric, Johansson [48] reported the behavioural differences between pigs that were on wet feed and dry feed. Accordingly, although pigs from both systems performed well, the researchers preferred the dry feed system considering a welfare-based standpoint. Dry feeding systems are gaining popularity and have been adopted by several farms as they require less labour [49]. Hence, feeding experiments were conducted employing green protein concentrate as a dry feed additive.

In this study, daily feed intake for the treatment diet was found to be better compared to the control. From the results presented in Table 6, it was evident that the weaner pigs on the treatment diet consumed more food as compared to the control, indicating that

the pigs preferred the treatment diet. This is indicative of the green protein imparting a better taste when incorporated in the feed. A recent study by Stødkilde, Ambye-Jensen [46] reported that adding green protein to the feed did not alter the taste to discourage the pigs from consuming it. Furthermore, addition of green protein from sources like clover grass improved the meat percentage and omega 3 fatty acids in meat.

Table 6. Crude fibre, crude protein and amino acid profile of oil seed meals [13].

Animal Feed Protein Sources	Crude Protein	Lysine	Methionine	Cysteine	Threonine	Crude Fibre
Soybean Meal	44–48	2.81–3.20	0.60–0.75	0.69–0.74	0.71–2.00	3.0–7.0
Sunflower Meal	24–44	1.18–1.49	0.74–0.79	0.55–0.59	1.21–1.48	12.0–32.0
Rapeseed Meal	36	2.00–2.12	0.67–0.75	0.54–0.91	1.53–2.21	10.0–15.0
Cottonseed Meal	24–41	1.05–1.71	0.41–0.72	0.64–0.70	1.32–1.36	25.0–30.0
Grass Protein Concentrate	33.9	1.81	0.65	0.18	1.5	6.1

Figures are represented as g per 100 g of feed.

4.3. Average Weight Gain

Variation in live weight and weight gain is undesirable in pig farming. It is essential to identify approaches that reduce the variation in live weight within a population compared to management approaches that attempt to minimize the impact of whatever level of variation exists [50]. In theory, it is only natural to observe variation in weights within a population at the end of finishing. Therefore, multiple approaches will need to be introduced to manage that variation. Reducing variation in growth rate is key in eliminating variation in live weight within the population. Some of the factors identified as influencers in weight variations among pigs include genetic factors, sex effects, birth weight, weaning age, feeding level and individual amino acid content (arginine levels) in the feed. Increasing the growth rate of all of the pigs in a population will not reduce variation but it will result in more of the lighter weight pigs reaching the minimum weight required by the market before the building needs to be emptied [51].

The average weight gain of pigs on the treatment diet was slightly lower than with that of weaners on control diet during the first week of the trial. This dip in AVG may be attributed to the need for the weaners to acclimatise themselves to the new diet. Interestingly, the AVG of the weaners on the treatment diet was found to be higher than that of control from week 2 of the trail. This trend continued until the end of the trial. The increase in AVG corresponds to the higher dry matter intake of weaner pigs on the treatment diet. A higher daily feed intake may have resulted in higher AVG. Consequently, the final weight gain achieved by the weaners on treatment diet was 6.44% higher than that of the control sample. The variation in average weight gain may be attributed to the inconsistency in the dry matter [49].

4.4. Feed Conversion Ratio

Feed conversion ratio is the measurement of the amount of feed required for the animal to gain one pound of body weight. A lower feed conversion ratio is an indication of the pigs efficiently turning feed into body weight. Several factors influence the feed efficiency in pigs. The amount of phosphorus fed to pigs should be maintained at an optimal level. Phosphorus in pig diets forms part of the structural compounds in bone and cell membrane and plays a vital role in energy metabolism and other metabolic pathways. Excess of phosphorus in the diet will result in the excretion of the mineral as faeces [50]. Pelleting of feed can improve the feed conversion ratio; quality pellets prevent the pigs from sorting and wasting the feed. Reduced pellet size can also assist digestion of the feed [51]. Finally, as was mentioned in the earlier section, lysine is the limiting amino acid in grain-based diets for pigs. Pigs require lysine in required amounts in order to effectively utilise other amino acids for growth [52].

From Table 6, the FCR values for the treatment and control diet were similar for the duration of the trial period except for the first week. This is an indication that the treatment diet is as efficient as the conventional diet fed to weaner pigs. The initial value of FCR for the treatment diet was higher than that of the control, perhaps due to the acclimatisation process required for the weaner pigs to digest the green protein. Nonetheless, from FCR values it can be observed that the conversion efficiency of the feed is not affected by the addition of the green protein concentrate. Additionally, the overall health of the pigs on the trial diet was similar to other pig groups reared on conventional diets [49].

5. Conclusions

A pig feed formulation which included green protein concentrate was found to be superior to soy-based pig diet. Characterisation of the green protein concentrate revealed that the crude protein content was comparable to that of soybean meal. All the indicators, i.e., the daily feed intake, feed conversion ratio and average daily weight gain, were consistently higher for the treatment diet compared to the control over the course of the feeding trials. This is an indication that the weaner pigs preferred the treatment diet over control. On the other hand, the lysine content in the green protein was not adequate enough to replace soybean meal in weaner diets. Overall, the results of the study, although preliminary, are quite promising and indicative of the potential of perennial ryegrass biorefined protein as a sustainable protein source for pig feed formulations. Further studies on the addition of this protein in pig feeds may include productive performances, ileal digestibility, etc., to further substantiate the efficacy of the green grass protein as a prospective feed protein source.

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Article

Biogas, Biomethane and Digestate Potential of By-Products from Green Biorefinery Systems

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Abstract: Global warming and climate change are imminent threats to the future of humankind. A shift from the current reliance on fossil fuels to renewable energy is key to mitigating the impacts of climate change. Biological raw materials and residues can play a key role in this transition through technologies such as anaerobic digestion. However, biological raw materials must also meet other existing food, feed and material needs. Green biorefinery is an innovative concept in which green biomass, such as grass, is processed to obtain a variety of protein products, value-added co-products and renewable energy, helping to meet many needs from a single source. In this study, an analysis has been conducted to understand the renewable energy potential of green biorefinery by-products and residues, including grass whey, de-FOS whey and press cake. Using anaerobic digestion, the biogas and biomethane potential of these samples have been analyzed. An analysis of the fertiliser potential of the resulting digestate by-products has also been undertaken. All the feedstocks tested were found to be suitable for biogas production with grass whey, the most suitable candidate with a biogas and biomethane production yield of 895.8 and 544.6 L/kg VS, respectively, followed by de-FOS whey and press cake (597.4/520.3 L/kg VS and 510.7/300.3 L/kg VS, respectively). The results show considerable potential for utilizing biorefinery by-products as a source for renewable energy production, even after several value-added products have been co-produced.

Keywords: green biorefinery; grass whey; anaerobic digestion; biogas; digestate

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1. Introduction

Climate change and interrelated environmental challenges represent the most eminent issues that threaten the future of the humankind. The European Commission launched 'The Green New Deal' in 2019, with aims to make Europe carbon neutral by 2050. As part of the deal, the EU has attributed more importance towards biodiversity, farming, energy and circular economy [1]. However, the energy sector is responsible for 75% of the total greenhouse emissions in the EU. Ensuring a greater contribution of renewable sources of energy across all sectors has been identified as a key building block for achieving climate and energy objectives. The Renewable Energy Directive (2009/28/EC) is a legal framework supporting renewable energy integration across all sectors in the EU [2]. This directive set out an overall target of 20% renewable energy integration by 2020, with binding contributory targets for each of the individual states. However, Ireland failed to meet its 2020 Renewable Energy Directive Target of 16%, falling short particularly in

areas of renewable heat and transport. As a result, Ireland now faces a tougher task of meeting its updated Renewable Energy Directive II (RED II) target of 32% by 2030 [3]. Furthermore, Ireland's Climate Action Bill requires a 51% reduction in GHG by 2030, rising to carbon neutrality by 2050. On the other hand, the agri-food sector is a vibrant industry in Ireland whose contribution to GDP has increased from 17.8% to 19.9% between 2019–2020 and 2020–2021 [4]. However, 30% of greenhouses emissions in Ireland are contributed by agricultural activities [5]. A recent report published by the Environmental Protection Agency in Ireland reported that methane levels in Ireland had increased by over 170% from the pre-industrial era, largely due to the livestock industry [6]. This calls for immediate and effective measures to reduce the environmental footprint of the Irish agriculture sector.

Biogas production has been identified as a potential step for the reduction in carbon emissions from primary production (e.g., livestock emissions and slurry related emissions) in recent years [7]. Biogas or biomethane is a renewable source of energy that can also directly reduce fossil-based emissions in sectors such as transport, heat and electricity, thus contributing to climate mitigation and renewable energy targets [8]. According to a survey conducted by the Central Statistics Office, Ireland, 3.7 million out of a total of 5 million hectares of agricultural land in Ireland, were grassland with 450,300 hectares attributed to rough grazing [9]. Grass silage has been explored as a potential source for anaerobic digestion [10–12]. However, operating an anaerobic digestion (AD) process following the mono-digestion of grass silage has been found to be unsustainable in the long term [13]. Interestingly, the co-digestion of silage with animal manures and slurries have been found to be a better alternative by comparison. According to a recent study published by Attard et al. (2020), more than 30 million tonnes of (wet weight) biomass were generated in Ireland in 2020, including 27 million tonnes of slurries and manures, while 1.7 million tonnes of dry matter (DM) of grassland is available annually beyond that are required to meet our food targets [14,15]. This 1.7 million could be increased to 12 million tonnes through the adoption of specific land management practices [15]. Several studies have investigated the co-digestion of grass and slurry. For example, Xie (2012) reported that maintaining pig manure and grass silage at a ratio of 3:1 yielded 304.2 mL CH_4/g ml when the two biomass sources were co-digested [16]. In another study, Himanshu et al. (2019) reported that maximum synergistic effects for biogas production can be achieved by maintaining a feed ratio of 0.75:0.25 for perennial ryegrass silages with cattle slurry [17]. Both studies observed that increasing the proportion of silage in the feedstock mixture progressively decreased the cost of methane production, increased the methane yield and increased overall plant profitability. A recent study conducted by Beausang et al. (2021) employed a consequential life cycle assessment to assess the environmental impacts of digesting different proportions of silage and cattle slurry for biogas production [18]. The study observed higher environmental burdens on the mixes with a greater ratio of grass silage to slurry with an optimum environmental performance observed at a VS ratio of 0.4:0.6 for silage and slurry, where there is a net reduction for all impact categories considered.

Ireland stands to gain from the large areas of grasslands and volumes of manure and slurries produced if these feedstocks can be effectively exploited for the production of biogas and biomethane via anaerobic digestion. The findings of Gas Networks Ireland claim that Ireland has the highest potential for biogas/biomethane production per capita in Europe, with a potential to achieve 13 TWh of energy via renewable gas by 2030. The agency has therefore put in motion a strategic plan to achieve 20% of renewable gas integrated within its network by 2030 [19]. Another interesting report published by KPMG suggested that replacing natural gas with biogas obtained using ADcan reduce carbon emissions by 93% [20]. Separately, O'Connor et al. (2021) examined the adoption of anaerobic digestion for energy production in Irish farms [21]. The study was conducted among cattle farmers to understand their motivations, perceived barriers and preferred business models. The study found that 41% of the farmers surveyed were supportive of installing an AD system on their farms. Lack of information regarding the technology and investment costs were

identified as the bottlenecks for AD installation on farms. Furthermore, approx. 60% of farmers were interested in adopting a co-operative business scheme to install and operate anaerobic digestors in their farms.

As we shift from a fossil-based to a bio-based economy, there is an increasing demand on our biological resources to meet our global food, feed and traditional material products, as well as energy requirements, while also providing an array of new products such as novel protein, bioplastics, chemicals and biofuels. A biorefinery can support more efficient use of biological resources, through co-processing into a range of bio-based products and energy. A green biorefinery is a sustainable process for exploiting green protein-rich biomass to produce a spectrum of commercially valuable products and energy. The feedstock for a green biorefinery can include any naturally occurring green biomass, such as grass, clover, sugar beet leaves, vegetable leaves etc. These plants and residues are rich in a wide variety of biomolecules, such as carbohydrates, lignin, proteins, lipids, polyphenols, andvitamins. The green biorefinery concept is at a relatively advanced stage in Western Europe, especially in countries such as Germany, Netherlands, Switzerland, Denmark and Austria [22]. Commercial green biorefinery enterprises have been developed by industry players, such as Biowert, Gramitherm and Newfoss, to produce plant-based products, such as technical fibers for composites, insulation materials, plastics and paper. At the same time, residual streams from the biorefinery process can be used to produce energy for the process, helping to improve the sustainability of the biorefinery. The Biowert Green Biorefinery cycle involves processing of grass silage to produce grass fibers, which are further converted into synthetic granules and biocomposite materials. The liquid that is a by-product of the biorefining process is used to produce biogas. The energy obtained from the AD process is sufficient enough to operate the biorefining process, while the digestate obtained after anaerobic digestion is further concentrated and converted into fertilizer, which is marketed to farmers completing the nutrient cycle [23]. Meanwhile, biorefineries that process grass feedstocks into plant protein feed and food products, as well as bioenergy, have also been developed by Aarhus University in Denmark and GRASSA B.V. in the Netherlands [24]. These approaches demonstrate the potential to use grass components to produce biogas through anaerobic digestion but follow a cascading biorefinery approach, which ensures the co-production of additional value-added co-products such as feed proteins and prebiotic ingredients. In addition to helping to meet Irelands renewable energy and climate obligations, such cascading approaches may also support the broader sustainability commitments of the United Nations' Sustainable Development Goals, helping to meet SDG7 (affordable and clean energy) by delivering a more economically viable co-processing approach, which balances the production of renewable energy, with higher value materials and proteins that are essential for animal and human consumption. Fonseca et al. (2020), highlights that SDG7 has significant relationships with other SDGs, including SDG1 (no poverty), SDG2 (zero hunger), SDG3 (good health and well-being), SDG8 (decent work and economic growth) and SDG13 (climate action) [25]. There, the wider impact of clean energy approaches, taking into account other pillars of sustainable living, is a key consideration.

A previous study on green biorefineries has been conducted in Denmark, where researchers evaluated a biorefinery process that utilised red clover, clover grass, alfalfa and oilseed radish as feedstocks for the extraction of protein for monogastric feed applications and subsequent conversion of press cake and grass juice into biogas and fertilizer [26]. The press cake and the brown juice were subjected to mono-digestion and co-digestion strategies. The study revealed that the co-digestion strategy was the preferred method for biogas production, considering ease in process control. Other work from Feng et al. (2021) explored the pilot-scale anaerobic digestion of by-product liquid (brown juice) from grass protein extraction using an un-heated anaerobic filter [27]. Using a different greenbiorefinery approach, Patterson et al. (2020) investigated the extraction of volatile fatty acids from grass and subsequent production of polyhydroxyalkanoates. In this case part of the unrefined grass was used for anaerobic digestion to produce biomethane [28].

Fernandez de Souza et al. (2020) explored the use of extrusion to pre-treat fresh grass and grass silage prior to anaerobic digesting and indicate enhanced biomethane yields of biogas of 18% and 11% respectively on a fresh matter basis [29]. The main objective of the current study was to assess the biogas and biomethane production potential of three biorefinery output streams that were generated as a result of a novel grass biorefining process. The current study presents and analyzes research of the Irish Biorefinery Glas green biorefinery project [30]. Perennial rye grass was used as a feedstock for the green biorefinery which enabled the extraction of grass protein concentrate and dietary fiber in the form of fructo-oligosaccharides (FOS). The residual streams of grass whey and de-FOS whey, in addition to a press cake co-product (otherwise used for feeding cows), were studied to understand their biogas potential. Finally, the digestate obtained from the different biogas production trials was also investigated as to understand the potential fertilizer value. While previous work has been undertaken in Ireland to assess the biomethane potential of unrefined perennial ryegrass [31–33], and some international studies have been conducted which analyze or estimate the biomethane potential of green biorefinery co-products [26,34], the authors could not find any Irish studies which have assessed the biogas or biomethane potential of green biorefinery co-products. In addition, the authors did not find any similar study in which the focus of research was a similar biorefinery sidestream (press cake, whey and de-FOS whey) resulting from the biorefinery of the feedstock perennial rye grass. Finally, the production of FOS is a novel aspect of the green biorefinery process used, so the application of de-FOS whey as a substrate for biogas production is assessed for the first time.

2. Materials and Methods

2.1. Green Biorefinery Residual Co-Products (Grass Press Cake, Grass Whey and De-FOS Whey) Preparation

Perennial rye grass (*Lolium perenne*) obtained from a farm located in West Cork, Republic of Ireland was utilized for input into a novel green biorefinery developed by GRASSA B.V. A schematic of the process is presented in Figure 1. The fresh grass was washed and then subjected to an extrusion process to obtain a high dry matter press cake fraction and a high protein juice fraction. The grass juice was then filtered and subjected to heat treatment at 80 °C, followed by centrifugation to remove the precipitated protein. The residual liquid fraction (whey) following the protein extraction was further processed to extract FOS, leaving a residual stream (de-FOS whey). The production of FOS is a unique feature of the biorefinery process, not included within similar fresh green biorefinery approaches described in the literature [34,35]. The press cake fraction, whey and de-FOS whey were analyzed for total solids, volatile solids, elemental analysis and fiber composition. All potential feedstocks were stored at 4 °C until further analysis. All analyses were completed within two weeks of the extraction process.

2.2. Biomethane Potential (BMP) Assays

All three assessed green biorefinery co-products (grass press cake, grass whey and de-FOS whey), in addition to unrefined ryegrass silage, were investigated for their biomethane potential (BMP). Unrefined ryegrass silage was included within the analysis for comparative purposes. The BMPs of the green biorefinery residual co-products were determined by employing an Anaero BMP system and followed protocols outlined in the German standard method VDI 4630. The BMP system consists of 15 plastic bottle digesters, each with a volume of one liter. BMP tests were performed with active inoculum maintained in the lab by feeding mixed waste streams (food waste, grass silage and whey permeate). The inoculum was sieved and degassed to avoid interference of organic matter present in the inoculum with the test sample. The volume to headspace ratio of the digester is 7:3, and the inoculum to substrate ratio is 4:1 for the BMP analysis. The pH of each digester was within the optimum anaerobic digestion range; hence, a pH adjustment with special reagents or buffers was not done in these BMP tests. All digesters were mixed at the same speed

using stainless steel paddle systems that guaranteed even mixing for all substrate-inoculum mixtures. The temperature of each digester was maintained at 37 °C with a water bath that incorporates a tight-fitting cover, which minimizes bathwater evaporation loss, even at thermophilic temperatures. All experiments were conducted in triplicates and lasted for 21 days of digestion.

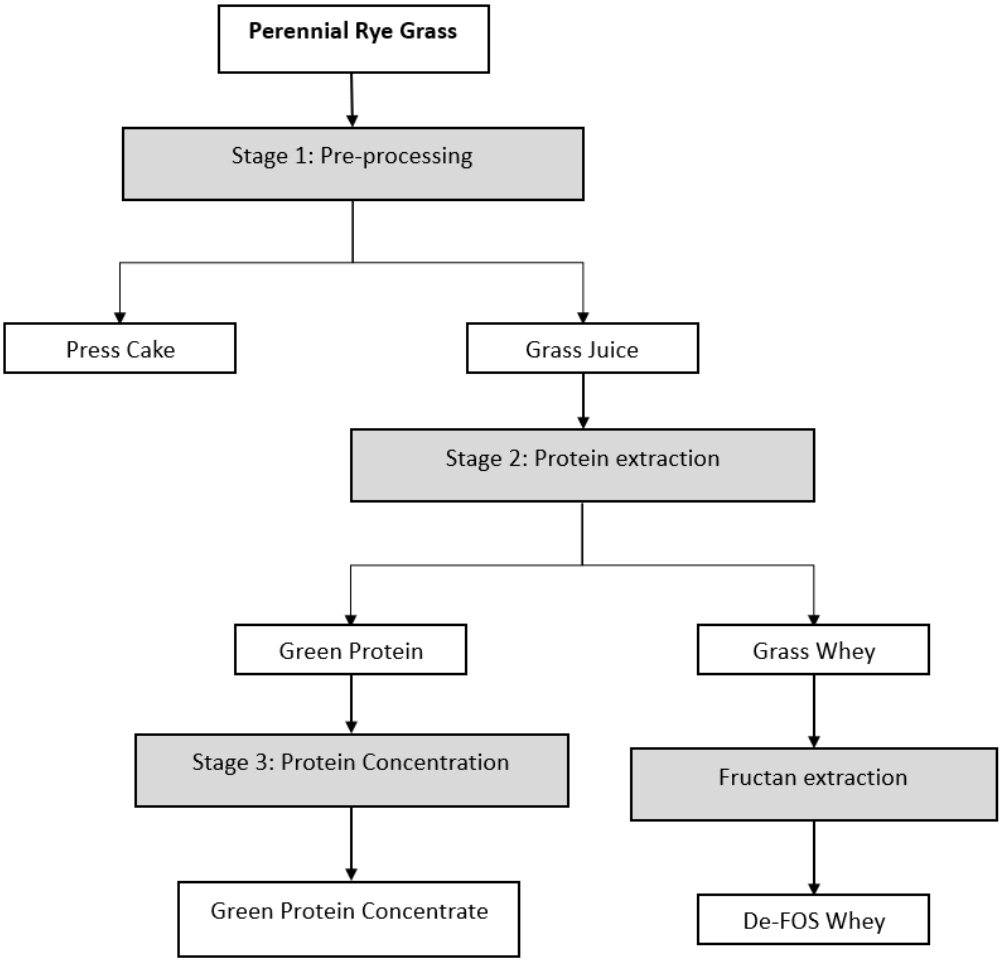

Figure 1. Schematic of green biorefinery process.

2.3. Compositional Analysis of Press Cake

Composition of the biorefinery press cake was analyzed by following the Laboratory Analytical Procedure (LAP), published by the National Renewable Energy Laboratory (NREL) [36]. In brief, the press cake was dried in the oven at 70 °C and milled to 0.2 um size using a Retsch Ultra Centrifugal Mill ZM 200. The milled sample was subjected to water extraction, followed by ethanol extraction (95% ethanol) at 100 °C and 1500 PSI using a Dionex accelerated solvent extraction system (ASE). The extractives obtained are classified as water, ethanol and full extractives (water followed by ethanol). The extracted solid residue is dried at 40 °C and hydrolyzed by two-step acid hydrolysis method. The hydrolysate is filtered through a medium-size filtering crucible, and the solid residue retained on the filter was kept for moisture and ash analysis to determine the acid insoluble residue and Klason lignin, respectively. Acid-soluble lignin in the filtrate is measured by recording the absorbance of liquid at 240 nm and applying the equation provided in the NREL LAP method. The sugars released by acid hydrolysis were analyzed by Ion chromatography with a PA1 column and melibiose as the internal standard.

2.4. Analytical and Chemical Methods

2.4.1. Chemical Characterization of Biorefinery Co-Product Substrates and Digestate

The analytical and chemical methods used for the characterization of the various biorefinery residual co-products followed protocols outlined in the European standards reference methods indicated in Table 1. All these analytical methods were also applied to corresponding digestates after biomethane potential tests. Proximate analysis (total solids, volatile solids, moisture and ash contents) of the substrates and corresponding digestates were measured according to the European standards outlined in the reference methods EN 14774-1:2009 and EN 14775:2009. The carbon, hydrogen, nitrogen and oxygen were analyzed with a vario MARCO cube elemental analyzer that used a thermal conductivity detector (TCD) and an optional infrared detector to determine the sulfur content of the substrates. The chemical oxygen demand (COD) estimates the amount of oxygen needed by organic matter present in the sample to be oxidized by a strong chemical oxidant. The COD was analyzed by first using a Hanna Instruments heating reactor (HI 839800 Reactor) at 150 °C for 2 h to digest the COD reagent kit (HI93754C-0) containing a 200 mg sample, and, afterward, a Hanna Instruments multiparameter photometer (HI 83399) was used to determine the COD the digested COD tubes. The Hanna Instruments multiparameter photometer utilized for the COD analysis was also used to determine the ammonia content of the various substrates. However, this protocol used an ammonia high range reagent kit (H193733-01) and does not involve a 2-h digestion step as outlined in the COD method. The biological oxygen demand (BOD) determines the amount of oxidizable organic matter by microorganisms in a unit value of the substrate. Approximately 1 g of the sample, together with a Hach BOD nutrient buffer (APHA formulation), were placed in an air-tight BOD serum bottle for five days at 20 °C. Readings were measured on the first and last day of the test with the Hanna Instruments conductivity meter (H1764080) and used to determine the BOD of the substrates. An Agilent ICP-OES 5110 with an axial view configuration spectrometer and Seaspray flow nebulizer was used to analyze for major and minor elements in digested substrates. Sample digestion was achieved in a "MARS 6 Microwave Reaction System" at 190 °C for 65 min and followed protocols outlined in reference method US EPA 3050. Furthermore, certified reference sample 1570 A in the form of spinach was also digested along with the biorefinery co-product samples to validate the quality of a subsequent ICP analysis.

Table 1. Feedstock analysis for biorefinery products.

Analysis	Reference Method	Units	Grass Press Cake	Grass Whey [#]	De-FOS Whey
Moisture	EN 14774-1:2009 (STANDARD, 2010)	%	61.00 *	97.97 *	91.33 *
Total Solids	Calculated	%	39.00 *	2.03 *	8.67 *
Ash	EN 14775:2009 (Standard, 2009)	%	4.67	20.99	19.94
Volatile Solids	Calculated	%	95.33	79.01	80.06
NPK			14:1:7.5	2.9:1:11.8	4.3:1:7.3
Carbon	EN 15104:2011 (EN, 2011 b) (ISO/TC 238, 2015 a)	%	47.81	37.73	40.70
Hydrogen	EN 15104:2011	%	5.73	5.09	4.76
Nitrogen	EN 15104:2011	%	2.74	2.18	4.34
Sulphur	EN 15289:2011(ISO/TC 238, 2015 a)	%	0.22	0.37	0.35
Oxygen	By Difference	%	38.84	33.64	29.90
Aluminium	EN ISO 16967:2015 (EN, 2011 a)	ppm	224 (87) *		313 (27) *
Calcium	EN ISO 16967:2015	ppm	4254 (1659) *		7977 (692) *
Iron	EN ISO 16967:2015	ppm	413 (161) *		387 (34) *
Magnesium	EN ISO 16967:2015	ppm	1100 (429) *		4973 (431) *
Sodium	EN ISO 16967:2015	ppm	2379 (928) *		2750 (238) *
Phosphorus	EN ISO 16967:2015	ppm	1960 (764) *	7434	10063 (872) *
Potassium	EN ISO 16967:2015	ppm	15159 (5912) *	87057	72877 (6318) *
Silicon	EN ISO 16967:2015	ppm	2434 (949) *		1821 (158) *
Titanium	EN ISO 16967:2015	ppm	12 (5) *		11 (1) *
COD	Modified EPA 410.4 (O'Dell, 1993)	g/kg	1143	968	984
BOD	In-House	g/kg	924	798	837
Ammonia	In-House	g/kg	1.54 *		1.62 *

[#] Proximate analysis was reported for 2-fold dilution of grass whey with washing water. Note: * values reported on as-received basis. All other values are reported on dry mass basis.

2.4.2. Biogas Production Potential

The biogas production from each digester was measured continuously by the flow meter provided with the Anaero system, and the gas was collected in 2 L Tedlar bags. The flow meter was composed of 15 chambers (each with a buoyancy bucket design) filled with a salt solution that did not allow the dissolution of carbon dioxide, hydrogen sulphide or ammonia content of the produced biogas. The gas volume is corrected for temperature and pressure to obtain normalized biogas volumes. The biogas collected in the Tedlar bags was analyzed for composition using a Biogas 5000 gas analyzer equipped with methane, carbon dioxide, hydrogen sulphide, ammonia and oxygen sensors. The gas composition analysis was done on the 3rd, 7th, 14th and 21st days of the BMP analysis. The biogas and biomethane yields for biorefinery feedstock were determined using equations provided below:

$$\text{Cumulative biogas produced} = \sum_{\text{Day0}}^{\text{Day21}} (\text{biogas produced per day (mL)}) \quad (1)$$

Ref. [37]

$$\text{Biogas yield} = \frac{\text{Cumulative biogas produced (mL)}}{\text{FM or TS or VS fed (g)}} \quad (2)$$

Ref. [38]

$$\text{Average } CH_4 \text{ percentage} = \text{Determined from Biogas analyzer 5000} \quad (3)$$

Ref. [39]

$$\text{Cumulative } CH_4 \text{ produced} = \sum_{\text{Day0}}^{\text{Day21}} (\%\text{Average } CH_4 \times \text{biogas produced per day (mL)}) \quad (4)$$

Ref. [40]

$$\text{Methane yield} = \frac{\text{Cumulative methane produced (L)}}{\text{FM or TS or VS fed (kg)}} \quad (5)$$

Ref. [41]

$$\text{Biodegradability } (B_d)\ \% = \frac{\text{Experimental methane yield } (EMY)}{\text{Theoretical methane yield } (TMY)} \times 100 \quad (6)$$

$$\text{Where } TMY = \frac{22.4 \times 1000 \times \left(\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8}\right)}{12a + b + 16c + 14d}$$

And a, b, c & d are subscripts for the empirical formula of substrate, i.e. $C_aH_bO_cN_d$ [42].

3. Results

3.1. Biomass Composition of the Green Biorefinery Press Cake, Grass Whey and De-FOS Whey

Feedstock analysis was performed for all the potential substrates (press cake, grass whey and de-FOS whey) derived from the greenbiorefinery for anaerobic digestion. The feedstock analysis involved proximate analysis (moisture and ash content), ultimate analysis (CHNOS, major and minor elements) and fiber content (Table 1). The moisture content in the press cake was found to be 61%. Alternatively, grass whey and de-FOS whey being liquids had a high moisture content of 98% and 91%, respectively. Meanwhile, the total solids accounted for 39% (*w/w*) of the press cake, 2.0% (*w/w*) for grass whey and 8.6% (*w/w*) for de-FOS whey, respectively. The volatile solids and ash content analyses for both the wet and dry samples of the press cake revealed that 37% of the total composition of wet samples were contributed by volatile solids, while this number was 95% for dry samples. Conversely, the volatile solids and ash content in the grass whey was 79% and 21%, respectively. Additionally, volatile solids and ash amounted to 6.9% and 80.1% of the

total weight of de-FOS whey, respectively. The ratio of volatile solids to total solids is an important parameter in the efficiency of anaerobic digestion and methane production [43].

Elemental analysis was performed to determine the carbon, nitrogen, hydrogen and sulfur content in the press cake, grass whey and de-FOS whey. The C:N ratios for grass press cake and grass whey were found to be 17.4:1 and 19:1, while the C:N ratio for de-FOS whey was determined to be 9.3:1. The C/S ratios in the grass press cake, grass whey and de-FOS whey were found to be 207.2:1, 76:1 and 116:1, respectively. Meanwhile, the BOD and COD analysis of grass whey were conducted to determine the biodegradability of the feedstock. The BOD amounted to 798 g/kg dry matter (DM), which amounted to 79% of the COD (968 g/kg DM). This was similar for de-FOS whey, which had a BOD of 837 g/kg DM and amounted to 85% of the measured COD of 984 g/kg DM.

Potassium and sodium contents in the feedstocks were also measured as part of the elemental analysis. Detailed figures of the elemental analysis of the feedstocks have been provided in Tables 1 and 2. The concentration of potassium and sodium were found to be 5912 ppm and 928 ppm, as well as 6318 ppm and 238 ppm (fresh basis) for press cake and de-FOS whey, respectively. The phosphorus concentration of all biorefinery feedstocks, meanwhile, ranged between 150 ppm and 900 ppm. At the same time, a trace elements analysis indicated that metals, such as iron, cobalt and molybdenum, were lower than required and would therefore need to be supplemented (Table 2).

Table 2. Concentration of trace elements in biorefinery feedstock.

Analysis	Units	Grass Press Cake	De-FOS Whey
Arsenic	ppm	<1	-
Cadmium	ppm	<1	<1
Cobalt	ppm	2	<1
Chromium	ppm	41	<1
Copper	ppm	10	10
Mercury	ppm	18	<1
Manganese	ppm	35	169
Molybdenum	ppm	2	2
Nickel	ppm	54	8
Lead	ppm	<1	3
Antimony	ppm	<1	<1
Vanadium	ppm	<1	<1
Zinc	ppm	47	88

All other values are reported on dry mass basis and followed reference method EN ISO 16968:2015 [30].

3.2. Biogas and Biomethane Potential of Biorefinery Residual Co-Products (Grass Press Cake, De-FOS and Grass Whey)

Assessment of the yield markers of the substrate samples, are presented in Table 3 and revealed that the biogas potential of grass whey and de-FOS whey, in particular, were quite promising. The biogas potential and biomethane potential of grass whey were found to be 895.8 L/kg VS (707.7 L/kg VS) and 544 L/Kg VS, respectively, although the substrate stream was diluted two-fold with wash water. Meanwhile, the biogas and biomethane potential of the de-FOS whey were found to be 597 L/Kg VS (478 L/Kg DM) and 520 L/Kg VS, respectively. The BMP analysis was performed on grass silage to compare the feedstocks with respect to their biogas and biomethane potential. Accordingly, grass silage yielded 479 L/Kg VS of biogas and a biomethane potential of 300–700 L/Kg VS. The daily and cumulative biomethane yield for grass press cake, grass whey and de-FOS whey, as well as silage, from the BMP tests have been presented in Figure 2.

Table 3. Biogas and biomethane summary data for grass press cake, whey and de-FOS whey.

		Grass Press Cake	Grass Whey	De-FOS Whey	Grass Silage	Dairy Whey
C:N ratio		19:1	17:1	9:1	17:1	-
Biogas and biomethane production (L/kg)	VS	510.7 (300.3) *	895.8 (544.6) *	597.4 (520.3) *	808.1 (479.0) *	(510–600) *
	DM	486.9 (286.2) *	707.7 (430.3) *	478.2 (416.5) *	737.2 (436.9) *	(280–330) *
	FM	189.9 (111.6) *	14.3 (8.7) *	41.5 (36.1) *	132.9 (78.8) *	-
Final weighted biogas composition	CH_4 (%)	58.8	60.8	87.1	59.3	-
	CO_2 (%)	43	39.1	14.8	41.1	-
	O_2 (%)	0.1	0.2	0	0	-
	H_2S (ppm)	17.6	6.7	87.7	4.2	-
	NH_3 (ppm)	0	3.7	113.9	0	-
Biodegradability	%	55	70	63	-	-

* Biomethane yield markers for both feedstocks. Biomethane yield for dairy whey was referenced from [33].

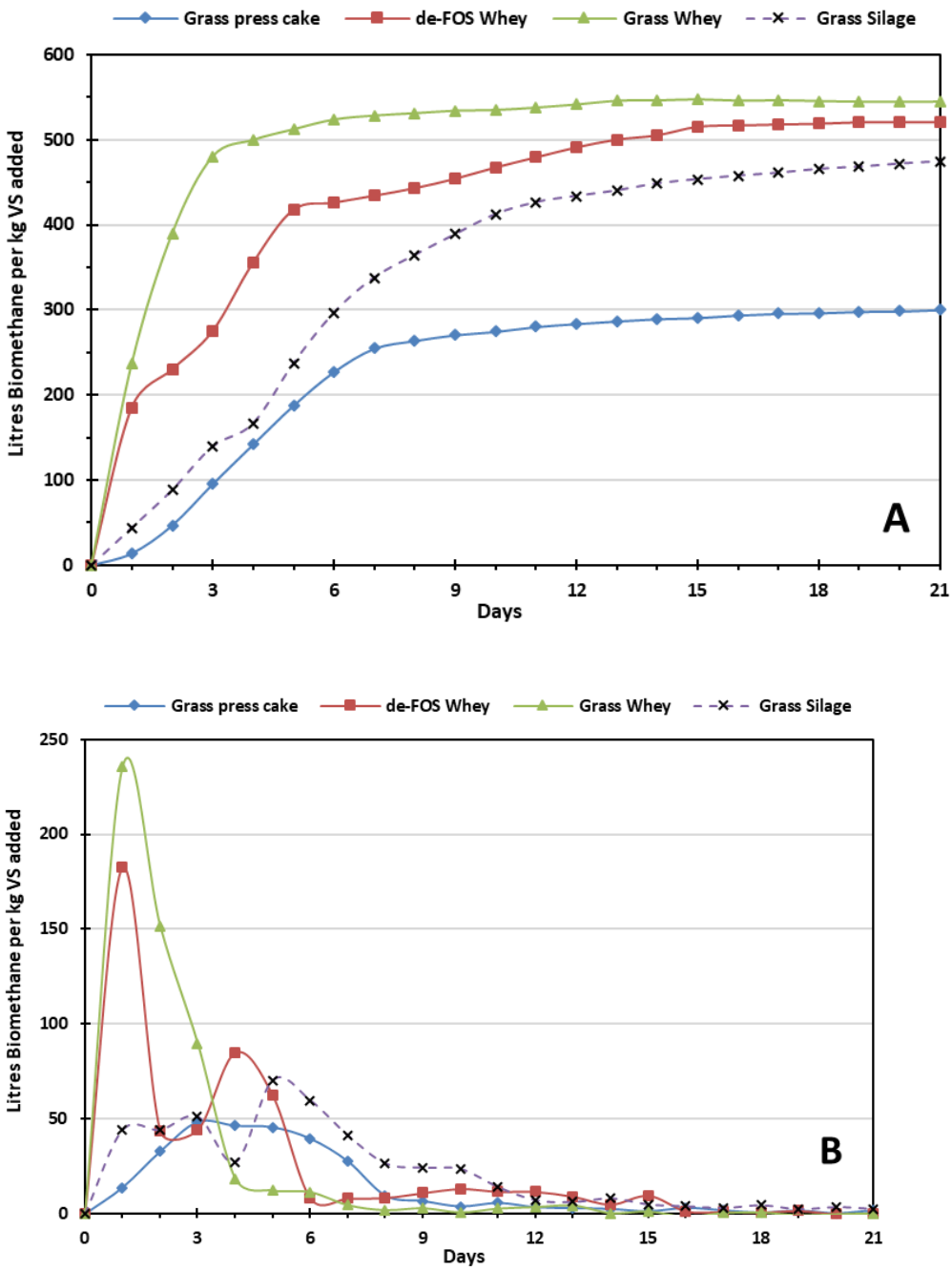

Figure 2. Cumulative (**A**) and daily (**B**) biomethane profiles for grass silage, grass press cake, whey and de-FOS whey.

3.3. Digestate Analysis to Determine the Fertilizer Potential of Biorefinery Residual Co-Products after Anaerobic Digestion

The digestate samples from anaerobic digestion of the various biorefinery products were analyzed and characterized for their potential to serve as biofertilizers for improving grassland soil (Table 4). A comparison of Tables 1 and 4 indicated a high volatile solids (VS) destruction of 70% for grass whey, with a low of 43% and 45% VS destruction for de-FOS whey and grass press cake, respectively. This was bolstered by a digestate ultimate analysis, which showed that the carbon content reduction in grass whey was significantly higher than that of grass press cake and de-FOS whey. The carbon to nitrogen (C:N) ratio for all digestate samples was either 8:1 or 9:1. The CODs and BODs of the various digestate samples were lower than the pre-digestion substrates. Another key factor that influences the suitability of a digestate as a biofertilizer is the nitrogen to phosphorous to potassium (NPK) ratio. The NPK ratios for grass press cake, whey and de-FOS whey digestates were 2:1.6:1,1:1.8:3 and 1.4:1:2, changing significantly from their pre-digestion values.

Table 4. Digestate analysis for biorefinery products.

Analysis	Units	Grass Press Cake	Grass Whey	De-FOS Whey
Moisture	%	97.60 *	98.99 *	96.47 *
Total Solids	%	2.40 *	1.01 *	3.53 *
Ash	%	49.31	76.36	54.02
Volatile Solids	%	50.69	23.64	45.98
C:N ratio		8:1	8:1	9:1
NPK ratio		2:1.6:1	1.4:1:2	1:1.8:3
Carbon	%	33.06	12.35	26.10
Hydrogen	%	3.88	1.66	4.31
Nitrogen	%	3.86	1.61	3.20
Sulphur	%	1.61	0.60	1.45
Oxygen	%	8.28	7.42	10.93
Aluminium	ppm	21,529		22,851
Calcium	ppm	47,502		56,109
Iron	ppm	30,548		31,798
Magnesium	ppm	6065		6277
Sodium	ppm	29,136		84,654
Phosphorus	ppm	31,838	11,788	59,756
Potassium	ppm	19,458	23,339	101,207
Silicon	ppm	9106		16,729
Titanium	ppm	383		620
COD	g/kg	833		906
BOD	g/kg	726		729
Ammonia	g/kg	0.52 *		1.57 *

Note: * values reported on as-received basis. All other values are reported on dry mass basis.

4. Discussion

4.1. Biomass Composition of the Green Biorefinery Press Cake, Grass Whey and De-FOS Whey

As discussed in Section 3.1., whey and de-FOS whey had much higher moisture contents compared to press cake. The moisture content is important in the removal of volatile solids in an anaerobic digestion process because the removal of VS decreases significantly with a lower moisture content. The ratio of volatile solids to total solids is an important parameter in the efficiency of anaerobic digestion and methane production [44]. The analysis found that the total solids accounted for 39% (*w/w*) of the press cake, 2.0% (*w/w*) for grass whey and 8.6% (*w/w*) for de-FOS whey, while volatile solids made up 95% of dry matter for press cake, 79% for grass whey and 80% for de-FOS whey.

The C:N is critical for determining whether the feedstock is suitable for mono-digestion or co-digestion. A high C:N ratio would result in the rapid consumption of nitrogen by acidogenic bacteria. Although this would positively affect the production of methane, the decrease in pH due to the accumulation of NH4+ ions would lead to low biogas production.

The optimal C:N ratio in feedstocks for methanogenesis in anaerobic digestors falls in the range of 10 to 30 [45]. As outlined in Section 3.1, the C:N ratio of the press cake and grass whey indicate that they can form an excellent feedstock for mono-digestion in the presence of necessary trace elements. The C:N ratio for de-FOS whey was determined to be 9.3:1, indicating that although this feedstock is suitable for monodigestion, the adoption of a co-digestion strategy with another carbon rich feedstock to maintain the C:N ratio within optimum range would be advantageous. Potentially suitable co-digestion feedstocks may include food waste and silage.

The presence of sulfur in biogas feedstocks has an adverse effect on the anaerobic digestion process due to the formation of H_2S which forms a constituent in the biogas produced. The recommended levels of H_2S in biogas must be lower than 100–500 mg/Nm^3. According to a study conducted by Peu et al. (2015), the carbon to sulfur ratio should be a minimum of 40:1 for the H_2S levels to be maintained below 2% (*v/v*) in the biogas [46]. The C/S ratio in the grass press cake, grass whey and de-FOS, outlined in Section 3.1, were found to be within these recommend levels, suggesting that the risk of H_2S is considerably low. Meanwhile, the BOD and COD analysis of grass whey and de-FOS whey indicated that all the volatile solids present in both samples were biodegradable, further signifying the suitability of this feedstock for anaerobic digestion.

The concentration of potassium in a potential feedstock for anaerobic digestion should ideally be greater than that of sodium to neutralize the cell membrane potential. Furthermore, high concentrations of these ions could potentially disrupt the biogas production process. In a study involving desugared molasses for anaerobic digestion, Fang et al. (2011) reported a 50% inhibition of the biogas production process at sodium and potassium concentrations of 11,012.56 and 28,032 ppm, respectively [47]. In the elemental analysis of the current study, the concentration of potassium and sodium in the press cake were found to be well below the inhibition concentrations. However, while the potassium levels were well below the inhibitory level when considering the dilution effect, the sodium concentration was found to be less than ideal. Furthermore, potassium levels were six times higher than that of sodium. A similar trend was observed for de-FOS whey, which had potassium and sodium levels of 6318 ppm and 238 ppm, respectively, with potassium levels of 26 times that of sodium. Interestingly, the levels of potassium were below the range of the inhibitory level when considering the dilution effect in the anaerobic digestion process.

Phosphorus is a key nutrient in the anaerobic digestion process; however, higher levels of phosphorus can lead to eutrophication, which is undesirable. At high concentrations (>500 ppm), it may inhibit the anaerobic digestion process thus detrimentally affecting efficiency [48]. The phosphorus concentration of all biorefinery feedstocks ranged between 150 ppm and 750 ppm. Therefore, considering the dilution effect in anaerobic digestion, the possibility of eutrophication and inhibitory effects of phosphorus can be eliminated in the digester.

4.2. Biogas and Biomethane Potential of Biorefinery Residual Co-Products (Grass Press Cake, De-FOS and Grass Whey)

Preliminary observations indicate that grass whey, de-FOS whey and grass press cake would not require pretreatment and should offer minimal process complications for biogas production. Although two and three separate products have been extracted from the whey and de-FOS whey, respectively, the biomethane potential of both residues were comparable to grass silage. In particular, grass whey showed quite good promise for biogas production, which may be enhanced with suitable co-digestion sources, as methane is a significant proportion of the overall gas volume, indicating that the biomethane potential of the feedstock is also significant. The biomethane potential of grass is also dependent on the cut of grass and harvesting stage [41].

Interestingly, the biomethane potential of de-FOS and grass whey were about 73% and 46% higher than that of grass press cake (300 L/Kg VS, 286 L/Kg DM) on a volatile solids and dry matter basis, respectively (Table 3). The higher biomethane potential of the liquid

feedstocks regardless of the lower volatile solid content (about 80% of DM as compared to press cake (95% of DM) may be attributed to the easily digestible solubilized components in both whey samples [49,50]. These solubilized components most likely account for a greater proportion of the 984 g/Kg COD measured for the whey. This is probably not the case with grass press cake, with the majority of its volatile solids content and the COD (1143 g/Kg) composed of complex components such as cellulose, hemicellulose, lignin and crude fiber. Interestingly, the biomethane potential of de-FOS and grass whey was similar to the dairy whey feedstock, which is reported to be 500 to 600 L/Kg VS on average (Table 3) [44]. This is encouraging, especially for de-FOS whey, since this feedstock was devoid of polysaccharides and proteins, as compared to dairy whey, which has high concentrations of disaccharide, lactose and protein [51]. A further observation of Figure 2 indicated that a short retention time below 5 days was able to produce 80% of the total biogas potential from both de-FOS and grass whey. The biogas productivities of grass whey and de-FOS whey were significantly better than grass silage which generally takes at least 12–20 days to yield such biogas productivity.

Methane on average accounted for 87% of total biogas produced from the de-FOS whey. De-FOS whey had excellent yield markers considering the BMP analysis of press grass cake and grass whey, yielding a weighted average methane content of 59%. However, the biomethane potential of de-FOS whey (36 L/Kg FM), as well as grass whey (8.7 L/Kg FM), on fresh matter basis was 68–93% lesser than that of grass press cake (112 L/Kg FM). This may be due to the high moisture contents of both whey substrates as compared to that of grass press cake (Table 1). Meanwhile, the high COD content of de-FOS and grass whey along with their shorter retention times for maximum biomethane production are desirable, as they are directly related to a reduction in capital cost and the increase in process efficiency. While an initial analysis indicates that most of the biogas could be produced within a number of days, this could feasibly decrease to a number of hours depending on the AD system, especially if high-rate digesters such as up flow anaerobic sludge blanket (UASB) and the induced blanket reactor (IBR) digesters are used for the digestion process [52].

Elemental analysis performed on grass press cake indicated a substrate with a good C:N ratio suitable for mono-digestion (Table 1). The biodegradability of grass press cake was lower (55%) than grass whey (63%). Regardless, grass press cake showed significant potential by achieving 80% of the total biogas after 7–10 days of digestion (Figure 2). Additionally, biomethane production from grass press cake achieved an efficiency of 71% based on predicted total methane from the COD analysis. Although this was lower than the efficiency of whey, the biodegradability index in terms of BOD5 to the COD ratio of 0.8 for grass press cake indicated that the majority of the sample was still biodegradable. Furthermore, on wet basis, the biomethane and biogas potential of grass press cake of 112 L/Kg and 190 L/Kg, respectively, were 3.1 and 4.5 times that of de-FOS whey (Table 3). With a biogas potential of 517 L/Kg VS and a biomethane potential of 304 L/Kg VS, the performance of grass press cake is about 37% less than that of grass silage (Table 3). Interestingly, fresh-basis biomethane yield from grass press cake was 30% higher than grass silage, making it a very interesting feedstock for biogas or biomethane production.

4.3. Digestate Analysis to Determine the Fertilizer Potential of Biorefinery Residual Co-Products after Anaerobic Digestion

The carbon to nitrogen (C:N) ratio for all digestate samples from digested biorefinery by-products was either 8:1 or 9:1 and was similar to that of soil organic matter (8:1 to 12:1) [53–55]. A higher C:N causes the depletion of nitrogen in the soil, as microorganisms strip the nitrogen from the soil to break down and assimilate carbon (microbial immobilization) [56]. However, the low C:N ratios observed for the various digestate samples indicated their suitability as fertilizer in terms of C:N ratio, as there is rapid mineralization and release of nitrogen in the soil for plant uptake [56]. In addition to the C:N ratio, the digestate BOD and biodegradability are two other factors considered to be key in affecting

the aerobic decomposition of organic matter in soil [57]. The CODs and BODs of the various digestate samples were lower than the pre-digestion substrates. The corresponding biodegradability index ranged within 0.80 and 0.87, indicating that the digestate samples were easily biodegradable under aerobic conditions in the presence of a suitable consortium bacteria, such as activated sludge [58].

The NPK for grass press cake digestate changed significantly with the anaerobic digestion (2:1.6:1) and was closer to the recommended NPK fertilizer concentration of 3:1:1 when compared to the other digestate samples resulting from digested grass whey and de-FOS whey [59]. The NPK for de-FOS whey digestate also changed significantly following anaerobic digestion (shifting from 4.3:1:7.3 to 1:1.8:3); however, the digestate NPK fell considerably below the recommended fertilizer NPK concentration of 3:1:1. A similar NPK ratio of 1.4:1:2 was also evaluated for grass whey digestate. Hence, both the de-FOS and grass whey digestate samples did not qualify to be used directly as organic fertilizer. However, due to their high potassium concentration, there is potential for them to be converted to potassium-rich fertilizer for potassium deficient soils. From the nitrogen, phosphorus and potassium levels, it was evident that the pre-digestion biorefinery substrates were not suitable for direct fertilizer use considering that their NPK ratios were beyond the recommended biofertilizer range of 3:1:1 (Table 1).

5. Conclusions

The paper has investigated and discussed the biogas and biomethane potential of three processing co-products and sidestreams resulting from a green biorefinery process based on perennial ryegrass as feedstock, namely press cake, whey and de-FOS whey. The analysis, taking into account key parameters such as the C:N ratio, C/S ratio, biodegradability of volatile solids, potassium and phosphorous levels, indicates that all three feedstocks are of suitable composition for use in anerobic digestion, including for mono-digestion. Grass whey and de-FOS whey yielded the highest biogas productivity with 895.8 L/kg VS and 597.4 L/kg VS, respectively. This was followed by press cake (510.7 L/kg VS). Meanwhile, de-FOS whey provided the highest overall yields of biomethane at 87% of total biogas. From the digestate analysis, it was clear that a large fraction of the volatile solids in the grass whey was consumed within the digestion process, justifying the high biogas production yield. Furthermore, the C:N ratio of all the feedstocks fell within the range of soil organic matter. However, only press cake digestate qualified as a potential fertilizer due to the NPK ratio falling within the recommended range. As for the grass whey and de-FOS whey digestates, the potassium content was found to be too high, beyond the recommended range, rendering them unsuitable as soil fertilizer. Further research is recommended to enhance nutrient concentration in the digestate by modifying the AD configuration to render them more suitable for agricultural purposes. Future research may also explore the further benefits of integrating green biorefinery with biogas production, including quantifying the environmental benefits of utilising residual heat and electricity from the AD system to meet the energy requirements of the process.

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